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PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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H. Raaphorst

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INNOGENETICS N.V.
B-9052 Gent
BELGIUM

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Purified hepatitis C virus single envelope proteins for diagnostic and therapeutic use

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INNOGENETICS N.V.
Industriepark Zwijnaarde 7, Box 4
B-9052 Gent/BE

PADR: 056560.6
INNOGENETICS N.V.
Patent Department, Industriepark Zwijnaarde 7, Box 4
B-9052 Gent
BE
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Hepatitis C virus single envelope proteins for diagnostic and therapeutic use

The present invention relates to the general fields of recombinant protein expression purification of recombinant proteins, synthetic peptides, diagnosis of HCV infection, prophylactic treatment against HCV infection and to the prognosis of the clinical effectiveness and/or clinical outcome of interferon therapy used to treat an individual with chronic hepatitis.

More particularly, the present invention relates to purification methods for hepatitis C virus envelope proteins, the use in diagnosis or therapy of HCV envelope proteins purified according to the methods described in the present invention, the use of single E1 and/or E2 envelope proteins in assays for monitoring disease and/or treatment of disease. The invention also relates to epitopes of the single E1 and/or E2 envelope proteins and monoclonal antibodies thereto.

The E2 protein purified from cell lysates according to the methods described in the present invention reacts with approximately 95% of patient sera. This reactivity is similar to the reactivity obtained with E2 secreted from CHO cells (Spaete et al., 1992). However, the intracellularly expressed form of E2 may more closely resemble the native viral envelope protein because it contains high mannose carbohydrate motifs, whereas the E2 protein secreted from CHO cells is further modified with galactose and sialic acid sugar moieties. When the aminoterminal half of E2 is expressed in the baculovirus system, about 13 to 21% of sera from several patient groups can be detected (Inoue et al., 1992). About 75% of HCV sera (and 95% of chronic patients) are anti-E1 positive using the purified, vaccinia-expressed recombinant E1 protein expressed intracellularly, in sharp contrast with the results of Kohara et al. (1992) and Hsu et al. (1993). Kohara et al. used a vaccinia-virus expressed E1 protein and detected anti-E1 antibodies in 7 to 23% of patients, while Hsu et al. only detected 14/50 (28%) sera using baculovirus-expressed E1. These results show that both a good expression system and purification protocol are required to reach a high reactivity of the single envelope proteins with human patient sera. This can be obtained using the proper expression system and purification protocols of the present invention which guarantee the conservation of the natural folding of the protein. After expression of E2 from E. coli, the reactivity of HCV sera was even lower and ranged from 14 (Yokosuka et al., 1992) to 17% (Mita et al., 1992). The amounts of purified protein needed for diagnostic screening assays are in the range of many grams per year. Also for vaccine purposes, high amounts of envelope protein would be needed. Therefore, the

vaccinia virus system may be used for selecting the best expression constructs and for limited upscaling, and large-scale expression and purification of single envelope proteins containing high-mannose carbohydrates may be achieved when expressed from several yeast strains. In the case of hepatitis B for example, manufacturing of HBsAg from mammalian cells was much more costly compared with yeast-derived hepatitis B vaccines.

It is an aim of the present invention to provide compositions comprising purified (single) recombinant E1 and/or E2 glycoproteins comprising conformational epitopes from the E1 and/or E2 domains of HCV.

It is another aim of the present invention to provide a new purification method for recombinantly expressed E1 and/or E2 proteins such that said recombinant proteins are directly usable for diagnostic and vaccine purposes as single recombinant proteins instead of polyproteins.

It is yet another aim of the present invention to provide novel recombinant vector constructs for recombinantly expressing E1 and/or E2 proteins, as well as host cells transformed with said vector constructs.

It is also an aim of the present invention to provide a method for producing and purifying recombinant HCV E1 and/or E2 proteins.

It is also an aim of the present invention to provide diagnostic and immunogenic uses of the recombinant HCV E1 and/or E2 proteins of the present invention, as well as to provide kits for diagnostic use or vaccines comprising any of the recombinant HCV E1 and/or E2 proteins of the present invention.

It is further an aim of the present invention to provide for a new use of E1 and E2 proteins, or suitable parts thereof, for monitoring / prognosing the response to interferon treatment of patients suffering from HCV infection.

It is also an aim of the present invention to provide E1 and/or E2 peptides which can be used for diagnosis of HCV infection and for raising antibodies.

It is also an aim of the present invention to provide monoclonal antibodies, more particularly humanized monoclonal antibodies, which react specifically with E1 and/or E2 epitopes comprising peptides.

It is also an aim of the present invention to provide kits for monitoring / prognosing the response of patients suffering from HCV infection to interferon treatment.

The following definitions serve to illustrate the different terms and expressions used in the present invention.

The term "hepatitis C virus single envelope protein" refers to a polypeptide or

polypeptide or analog (e.g. mimitopes) comprising an amino acid sequence (and/or amino acid analogs) defining at least one HCV epitope of either the E1 or the E2 region. Typically, the sequences defining the epitope correspond to the amino acid sequence of either the E1 or the E2 region of HCV (either identically or via substitution of analogs of the native amino acid residue that do not destroy the epitope). In general, the epitope-defining sequence will be 5 or more amino acids in lenght, more typically 8 or more amino acids in lenght, and even more typically 10 or more amino acids in lenght. With respect to conformational epitopes, the lenght of the epitope defining sequence can be subject to wide variations, since it is believed that these epitopes are formed by the three-dimensional shape of the antigen (e.g. folding). Thus, the amino acids defining the epitope can be relatively few in number, but widely dispersed along the length of the molecule being brought into the correct epitope conformation via folding. The portions of the antigen between the residues defining the epitope may not be critical to the conformational structure of the epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g. cysteines involved in disulfide bonding, glycosylation sites, etc.).

The HCV antigens of the present invention comprise conformational epitopes from the E1 and/or E2 (envelope) domains of HCV. The E1 domain, which is believed to correspond to the viral envelope protein, is currently estimated to span amino acids 192-383 of the HCV polyprotein (Hijikata et al., 1991, PNAS). Upon expression in a mammalian system (glycosylated), it is believed to have an approximate molecular weight of 35 kDa as determined via SDS-PAGE. The E2 protein, previously called NS1, is believed to span amino acids 384-809 of the HCV polyprotein and to also be an envelope protein. Upon expression in a vaccinia system (glycosylated), it is believed to have an apparent gel molecular weight of about 72 kDa. It is understood that these protein endpoints are approximations (e.g. the carboxy terminal of E2 could lie somewhere in the 730-820 amino acid region, e.g. ending at amino acid 730, 735, 740, 742, 744, 745, 746, 747, 748, 750, 760, 770, 780, 790, 800, 809, 810, 820). It is also understood that the isolates used in the examples section of the present invention were not intended to limit the scope of the invention and that any HCV isolate from type 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or any other new genotype of HCV is a suitable source of E1 and/or E2 sequence for the practice of the present invention.

The E1 and E2 antigens used in the present invention may be full-length viral

proteins, substantially full-length versions thereof, or functional fragments thereof (e.g. fragments which are not missing sequence essential to the formation or retention of a conformational epitope). Furthermore, the HCV antigens of the present invention can also include other sequences that do not block or prevent the formation of the conformational epitope of interest. The presence or absence of a conformational epitope can be readily determined though screening the antigen of interest with an antibody (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any). In such screening using polyclonal antibodies, it may be advantageous to adsorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest.

The HCV antigens of the present invention can be made by any recombinant method that provides the conformational epitope of interest. For example, recombinant intracellular expression in mammalian or insect cells is a preferred method to provide glycosylated E1 and/or E2 antigens in "native" conformation as is the case for the natural HCV antigens. Yeast cells and mutant yeast strains (e.g. mnn 9 mutant or glycosylation mutants derived by means of vanadate resistence selection) may be ideally suited for production of secreted high-mannose-type sugars; whereas proteins secreted from mammalian cells may contain modifications including galactose or sialic acids which may be undesirable for certain diagnostic or vaccine applications. However, it may also be possible, as it is known for proteins, to express the antigen in other recombinant hosts and renature the protein after recovery.

The term "fusion polypeptide" intends a polypeptide in which the HCV antigen(s) are part of a single continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or be separated by intervening amino acid sequences. The fusion polypeptides may also contain amino acid sequences exogenous to HCV.

The term "solid phase" intends a solid body to which the individual HCV antigens or the fusion polypeptide comprised of HCV antigens are bond covalently or by noncovalent mean such as hydrophobic adsorption.

The term "biological sample" intends a fluid or tissue of a mammalian individual (e.g. an anthropoid, a human) that commonly contains antibodies produced by the individual, more particularly by antibodies against HCV. Such components are known in the art and include, without limitation, blood, plasma, serum, spinal fluid, lymph fluid,

secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk, white blood cells and myelomas. Body components include biological liquids. The term "biological liquid" refers to a fluid obtained from an organism. Some biological fluids are used as a source of other products, such as clotting factors (e.g. Factor VIII;C), serum albumin, growth hormone and the like. In such cases, it is important that the source of biological fluid be free of contamination by virus such as HCV.

The term "immunologically reactive" means that the antigen in question will react specifically with anti-HCV antibodies present in a body component from an HCV infected individual.

The term "immune complex" intends the combination formed when an antibody binds to an epitope on an antigen.

"E1" as used herein refers to a protein or polypeptide expressed within the first 400 amino acids of an HCV polyprotein, sometimes referred to as the E or S protein. In its natural form it is a 35 kDa glycoprotein which is found in strong association with membrane. In most natural HCV strains, the E1 protein is encoded in the viral polyprotein following the C (core) protein. The E1 protein extends from approximately amino acid (aa) 192 to about aa 383 of the full-length polyprotein. The term "E1" as used herein also includes analogs and truncated forms that are immunologically cross-reactive with natural E1.

"E2" as used herein refers to a protein or polypeptide expressed within the first 900 amino acids of an HCV polyprotein, sometimes referred to as the NS1 protein. In its natural form it is a 72 kDa glycoprotein that is found in strong association with membrane. In most natural HCV strains, the E2 protein is encoded in the viral polyprotein following the E1 protein. The E2 protein extends from approximately aa 384 to about aa 809. The term "E2" as used herein also includes analogs and truncated forms that are immunologically cross-reactive with natural E2.

The term "homooligomer" as used herein refers to a complex of E1 and/or E2 containing more than one E1 or E2 monomer, e.g. E1/E1 dimers, E1/E1/E1 trimers or E1/E1/E1/E1 tetramers and E2/E2 dimers, E2/E2/E2 trimers or E2/E2/E2/E2 tetramers are all "homooligomers" within the scope of this definition. The oligomers may contain one, two, or several different monomers of E1 or E2 obtained from different types or subtypes of hepatitis C virus including for example those described in PCT/EP 94/01323. Such mixed oligomers are still homooligomers within the scope of this invention. Heterooligomers like the E1/E2 heterodimer are not included within the scope of this

invention.

The term "purified" as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, withouth affecting the determination of the percentage purity as used herein. An "isolated" HCV protein intends an HCV protein composition that is at least 35% pure.

The term "purified protein" refers to a protein which is substantially freed from other HCV viral components, particularly genomic HCV polynucleotide. A protein composition is "substantially free" of another component if the weight of the protein in the composition is at least 80% of the weight of the protein and other component combined, more preferably at least about 89%, still more preferably about 90%, more preferably 95% and more, and most preferably the amount of contaminating protein is undetectable.

The term "lower eukaryote" refers to host cells such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular. Preferred lower eukaryotes are yeasts, particularly species within Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia (e.g. Pichia pastoris), Hansenula (e.g. Hansenula polymorpha) and the like. Saccharomyces cerevisiae, S. carlsbergensis and K. lactis are the most commonly used yeast hosts, and are convenient fungal hosts.

The term "higher eukaryote" refers to host cells derived from higher animals, such as mammals, reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells), baby hamster kidney (BHK), pig kidney (PK15) rabbit kidney 13 cells (RK_{13}), the human osteosarcoma cell line 143 B, human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. Spodoptera frugiperda). The host cells may be provided in suspension or flask cultures, tissue cultures, organ cultures and the like.

The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides

containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term "recombinant polynucleotide" intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation : (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

The term "recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

The term "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

The term "vector" is a replicon further comprising sequences providing replication and/or expression of the open reading frame.

The term "control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

The term "promoter" is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent structural gene.

The expression "operably linked" refers to a juxtaposition wherein the components

so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

As used herein, "epitope" or "antigenic determinant" means an amino acid sequence that is immunoreactive. Generally an epitope consists of at least 3 to 5 amino acids, and more usually, consists of at least about 8, or even about 10 amino acids. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof.

The term "immunogenic" refers to the ability of a substance to cause a humoral and/or cellular response, whether alone or when linked to a carrier, in the presence or absence of an adjuvant. "Neutralization" refers to an immune response that blocks the infectivity, either partially of fully, of an infectious agent. A "vaccine" is an immunogenic composition capable of eliciting protection against HCV, whether partial or complete, useful for treatment of an individual.

The term "effective amount" refers to an amount of epitope-bearing polypeptide sufficient to induce an immunogenic response in the individual to which it is administered, or to otherwise detectably immunoreact in its intended system (e.g., immunoassay). Preferably, the effective amount is sufficient to effect treatment, as defined above. The exact amount necessary will vary from application. For vaccine applications or in the generation of polyclonal antiserum / antibodies, for example, the effective amount may vary depending on the species, age, and general condition of the individual, the severity of the condition being treated, the particular polypeptide selected and its mode of administration, etc. It is also believed that effective amounts will be found within a

relatively large, non-critical range. An appropriate effective amount can be readily determined using only routine experimentation.

The present invention contemplates a composition comprising essentially purified HCV single envelope proteins selected from the group consisting of E1 and E2, characterized as being isolated in the presence of a means for cleaving disulphide bonds and preferably also in the presence of a means for blocking disulphide bond formation. Said essentially purified HCV single proteins are further characterized in that they are free from contaminating proteins. Said essentially purified HCV single envelope proteins are preferentially also characterized in that they are separated from contaminating agents before or after said cleavage of the disulphide bonds as detailed below.

The term "essentially purified" refers to proteins purified such that they can be used for in vitro diagnostic methods and as therapeutic compound. Usually the proteins are purified to homogeneity (at least 80% pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%, and most preferably the contaminating proteins should be undetectable by conventional methods like SDS-PAGE and silver staining.

The term "single envelope proteins" refers to the fact that said proteins are recombinantly expressed as single unities, being either E1 monomers or oligomers or either E2 monomers or oligomers.

The term "recombinantly expressed" refers to the fact that said proteins are produced by recombinant expression methods be it in lower or higher eukaryotes as discussed in detail below. The proteins according to the present invention are preferably glycosylated and may contain high-mannose-type, hybrid, or complex glycosylations. Preferentially said proteins are expressed from mammalian cell lines as discussed in detail in the Examples section, or alternatively may be expressed from mutant yeast strains as discussed below.

The proteins according to the present invention may be secreted or expressed within components of the cell, such as the ER or the Golgi Apparatus. Preferably, however, the proteins of the present invention bear high-mannose-type glycosylations and are retained in the ER or Golgi Apparatus of mammalian cells or are retained or secreted from yeast cells, preferably secreted from yeast mutant strains such as the mnn9 mutant, or from mutants that have been selected by means of vanadate resistence. Upon expression of single HCV envelope proteins, it is shown in the present invention that some of the free thiol groups of cysteines not involved in intramolecular disulphide bridges, react with

cysteines of host or expression-system-derived (e.g. vaccinia) proteins or of other envelope proteins, and form intermolecular bridges. This results in the formation of "aggregates" of HCV envelope proteins and contaminating proteins. It was also shown in WO 92/08734 that "aggregates" were obtained after purification, but it was not described which protein interactions were involved. In patent application WO 92/08734, recombinant E1/E2 protein expressed with the vaccinia virus system were partially purified as aggregates and only found to be 70% pure. Therefore a major aim of the present invention resides in the separation of single HCV envelope proteins from contaminating proteins, and to use the purified proteins (> 95% pure) for diagnostic and therapeutic purposes. To those purposes, the invention provides evidence that aggregated protein complexes ("aggregates") are formed on the basis of disulphide bridges and non-covalent protein-protein interactions. The present invention thus provides a means for selectively cleaving the disulphide bonds under specific conditions and for separating the cleaved proteins from contaminating proteins which greatly interfere with diagnostic and therapeutic applications. The free thiol groups may be blocked (reversibly or irreversibly) in order to prevent the reformation of disulphide bridges, or may be left to oxidize and oligomerize with other envelope proteins (see definition homooligomer). It is to be understood that such protein oligomerization is structurally essentially different the "aggregates" described in WO 92/08734 and WO 94/01778, since the level of contaminating proteins is undetectable.

Said disulphide bond cleavage step may be carried out in the presence of a suitable detergent able to dissociate the expressed proteins, such as DecylPEG, EMPIGEN-BB, NP-40, sodium cholate, Triton X-100. Said disuphide bond cleavage may also be achieved by
(1) performic acid oxidation by means of cysteic acid in which case the cysteine residues are modified into cysteic acid (Moore et al., 1963).
(2) Sulfitolysis (R-S-S-R → 2 R-SO^-_3) for example by means of sulphite (SO^{2-}_3) together with a proper oxidant such as Cu^{2+} in which case the cysteine is modified into S-sulpho-cysteine (Bailey and Cole, 1959).
(3) Reduction by means of mercaptans, such as dithiotreitol (DDT), ß-mercapto-ethanol, cysteine, glutathione Red, ε-mercapto-ethylamine, or thioglycollic acid, of which DTT and ß-mercapto-ethanol are commonly used (Cleland, 1964), is the preferred method of this invention because the method can be performed in a water environment and because the cysteine remains unmodified.
(4) Reduction by means of a phosphine (e.g. Bu_3P) (Ruegg and Rudinger, 1977).

A preferred reducing agent according to the present invention is dithiothreitol (DTT).

Partial reduction is obtained by using a low concentration of said reducing agent, i.e. for DTT for example in the concentration range of 0.1 to 50 mM, preferably 0.1-20 mM, preferably 0.5 to 10 mM, preferably 1.5 mM, 2.0 mM, 2.5 mM, 5 mM or 7.5 mM.

Said partial reduction is carried out preferably in in the presence of a detergent. A preferred detergent according to the present invention is Empigen-BB. The amount of detergent used is preferably in the range of 1 to 10 %, preferably 3% of a detergent such as Empigen-BB.

As contemplated in the Examples section, the particular combination of a low concentration of DTT and about 3 % of Empigen-BB is proven to be a particularly preferred combination of reducing agent and detergent for the purification of recombinantly expressed vaccinia E1 and E2 proteins. Upon gelfiltration, said partial reduction is shown to result in the production of dimeric E1 protein and separation from contaminating proteins that cause false reactivity upon use in immunoassays.

It is, however, to be understood that any other combination of any reducing agent known in the art with any detergent or other means known in the art to make the cysteines better accessible is also within the scope of the present invention, insofar as said combination reaches the same goal of disulphide bridge cleavage as the preferred combination examplified in the present invention.

Apart from reducing the disulphide bonds, a disulphide bond cleaving means according to the present invention may also include any disulphide bridge exchanging agents (competitive agent being either organic or proetineaeous, see for instance Creighton, 1988) known in the art which allows the following type of reaction to occur:

$$R1\ S-S\ R2 + R3\ SH \rightarrow R1\ S-S\ R3 + R2\ SH$$

* R1, R2: compounds of protein aggregates
* R3 SH: competitive agent (organic, proteinaeous)

Another aspect of the present invention includes the use of any SH group blocking or binding reagent known in the art such as:

- Glutathion
- 5,5'-dithiobis-(2-nitrobenzoic acid) or bis-(3-carboxy-4-nitrophenyl)-disulphide (DTNB or Ellman's reagent) (Elmann, 1959)
- N-ethylmaleimide (Benesch et al., 1956)
- N-(4-dimethylamino-3,5-dinitrophenyl) maleimide or Tuppy's maleimide which provides a color to the protein
- P-chloromercuribenzoate (Grassetti et al., 1969)

- 4-vinylpyridine (Friedman and Krull, 1969) can be liberated after reaction by acid hydrolysis
- acrylonitrile, can be liberated after reaction by acid hydrolysis (Weil and Seibles, 1961)
- NEM-biotin
- 2,2'-dithiopyridine (Grassetti and Murray, 1967)
- 4,4'-dithiopyridine (Grassetti and Murray, 1967)
- 6,6'-dithiodinicontinic acid (DTDNA; Brown and Cunnigham, 1970) (DTDNA)
- 2,2'-dithiobis-(5'-nitropyridine) (DTNP; US patent 3597160) or other dithiobis (heterocyclic derivative) compounds (Grassetti and Murray, 1969)

A survey of the publications cited shows that often different reagents for sulphydryl groups will react with varying numbers of thiol groups of the same protein or enzyme molecule. One may conclude that this variation in reactivity of the thiol groups is due to the steric environment of these groups, such as the shape of the molecule and the surrounding groups of atoms and their charges, as well as to the size, shape and charge of the reagent molecule or ion. Frequently the presence of adequate concentrations of denaturants such as sodium dodecylsulfate, urea or guanidine hydrochoride will cause sufficient unfolding of the protein molecule to permit equal access to all of the reagents for thiol groups. By varying the concentration of denaturant, the degree of unfolding can be controlled and in this way thiol groups with different degrees of reactivity may be revealed. Although up to date most of the work reported has been done with p-chloromercuribenzoate, N-ethylmaleimide and DTNB, it is likely that the other more recently developed reagents may prove equally useful. Because of their varying structures, it seems likely, in fact, that they may respond differently to changes in the steric environment of the thiol groups

Alternatively, conditions such as low pH for preventing free SH groups from oxidizing and thus preventing the formation of large intermolecular aggregates upon recombinant expression and purification of E1 and E2 (envelope) proteins are also within the scope of the present invention.

A preferred SH group blocking reagent according to the present invention is N-ethylmaleimide (NEM). Said SH group blocking reagent may be administrated during lysis of the recombinant host cells and after the above-mentioned partial reduction process or after any other process cleaving disulphide bridges. Said SH group blocking reagent may also be modified with any group capable of providing for a detectable label and/or any

group aiding in the immobilization of said recombinant protein to a solid substrate, e.g. biotinylated NEM.

Methods for cleaving cysteine bridges and blocking free cysteines have also been described in Darbre (1987), Means and Feeney (1971), and by Wong (1993).

The present invention also contemplates a recombinant vector comprising a vector sequence, an appropriate (prokaryotic), eukaryotic or viral promoter sequence followed by a nucleotide sequence allowing the expression of the single E1 or E2 proteins of the invention. The segment of the HCV cDNA encoding the desired E1 and/or E2 sequence inserted into the vector sequence may be attached to a signal sequence. Said signal sequence may be that from another source, but particularly preferred constructs according to the present invention contain signal sequences appearing in the HCV genome before the respective start points of the E1 and E2 proteins. The segment of the HCV cDNA encoding the desired E1 and/or E2 sequence inserted into the vector may also include a deletion of the hydrophobic domain(s) as illustrated in the examples section.

More particularly, the present recombinant vector encompasses a recombinant nucleic acid having a HCV cDNA segment starting in the region between positions 117 and 192, and ending at any position in the region between positions 263 and 326, for expression of HCV single E1 protein, or forms that have the putative membrane anchor deleted (positions 264 to 293 plus or minus 8 amino acids).

A variety of vectors may be used to obtain recombinant expression of HCV single envelope proteins. Lower eukaryotes such as yeasts and glycosylation mutant strains are typically transformed with plasmids, or are transformed with a recombinant virus. The vectors may replicate within the host independently, or may integrate into the host cell genome.

Higher eukaryotes may be transformed with vectors, or may be infected with a recombinant virus, for example a recombinant vaccinia virus. Techniques and vectors for the insertion of foreign DNA into vaccinia virus are well known in the art, and utilize, for example homologous recombination. A wide variety of viral promoter sequences, possibly terminator sequences and poly(A)-addition sequences, possibly enhancer sequences and possibly amplification sequences, all required for the mammalian expression, are available in the art. Vaccinia is particularly preferred since vaccinia halts the expression of host cell proteins. Vaccinia is also very much preferred since it allows the expression of E1 and E2 proteins of HCV in cells or individuals which are immunized with the live recombinant vaccinia virus. For vaccination of humans the avipox and Ankara Modified Virus (AMV) are

particularly useful vectors.

Also known are insect expression transfer vectors derived from baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV), which is a hepler-independent, viral expression vector. Expression vectors derived from this system usually use the strong viral polyhedrin gene promoter to drive the expression of heterologous genes. Different vectors as well as methods for the introduction of heterologous DNA into the desired site of baculovirus are available to the man skilled in the art for baculovirus expression. Also different signals for posttranslational modification recognized by insect cells are known in the art.

Said recombinant vector includes a recombinant polynucleotide which also forms part of the present invention. The present invention relates more particularly to the recombinant nucleic acids as represented in SEQ ID NO 3, 4, 5, 6, 7, 8, 15, 16, 17, 18, 19, 20, 23, 24, 25, 26, 27, 28, 29 or 30.

As illustrated extensively by the present examples, vaccinia virus is a preferred expression system for the proteins according to the present invention.

The present invention also contemplates host cells transformed with a recombinant vector as defined above, wherein said vector comprises a nucleotide sequence encoding HCV E1 and/or E2 protein as defined above in addition to a regulatory sequence operably linked to said HCV E1 and/or E2 sequence and capable of regulating the expression of said HCV E1 and/or E2 protein.

Eukaryotic hosts include lower and higher eukaryotic hosts as described in the definitions section. Lower eukaryotic hosts include yeast cells well known in the art. Higher eukaryotic hosts mainly include mammalian cell lines known in the art and include many immortalized cell lines available from the ATCC, inluding HeLa cells, Chinese hamster ovary (CHO) cells, Baby hamster kidney (BHK) cells, and a number of other cell lines.

The present invention particularly relates to a method for producing and purifying HCV single envelope protein selected from the group consisting of E1 and E2, comprising the essential step of incubation with a disulphide bond cleaving agent as detailed above, and preferably also incubation with an (disulphide group (re)forming agent) SH group blocking agent also as detailed above. Preferably said HCV single envelope proteins are purified by means of lectin-chromoatography or by means of immunoaffinity using E1 and/or E2 specific monoclonal antibodies. A preferred lectin-chromatography system is lentil lectin chromatography as illustrated in the Examples section.

Preferably said method is usable to purify single HCV envelope protein produced

intracellularly as detailed above.

The method of the present invention for producing HCV single envelope proteins comprising the essential step of incubation with a disulphide bond cleaving agent, and preferably also incubation with a disulphide bond (re)forming agent.

A preferred method as defined above is further characterized as comprising the following steps:

- growing a lower or higher eukaryote host cell as defined in claim 4 transformed with a recombinant vector according to any of claims 2 to 3 in a suitable culture medium,
- causing expression of said vector sequence as defined in any of claims 2 to 3 under suitable conditions, and,
- recovering said recombinant HCV protein from said cell culture by lysing said cells, preferably in the presence of a SH group blocking agent, such as N-ethylmaleimide (NEM),
- recovering said HCV single envelope protein by affinity purification by means of lectin-chromatography or immunoaffinity chromatography using anti-E1 and/or anti-E2 specific monoclonal antibodies, with said lectin being preferably lentil-lectin, followed by,
- incubation of the eluate of the previous step with a disulphide bond cleavage means, such as DTT, preferably also in the presence of an SH group blocking agent, such as NEM or Biotin-NEM, and,
- isolating the HCV single E1 and/or E2 proteins by means of gelfiltration or Ni-IMAC.

As a result of the above-mentioned proces, E1 and E2 protein may be produced in a stable dimeric form which elutes different from the large aggregates containing vaccinia components in the void volume of the gelfiltration column or the IMAC collumn as illustrated in the Examples section. The disulphide bridge cleavage step advantageously also eliminates the false reactivity due to the presence of host or expression-system-derived proteins.

NiIMAC chromatography is preferably used for contructs bearing a $(His)_6$ as described by Janknecht et al., 1991 and Hochuli et al., 1988.

The present invention further relates a composition comprising at least one of the following E1 peptides as listed in Table 3:

Env 31 or E1-31 (SEQ ID NO 36) spanning amino acids 181 to 200 of the E1 region,

Env 35 or E1-35 (SEQ ID NO 38) spanning amino acids 205 to 224 of the E1 region,

Env 35A or E1-35A (SEQ ID NO 39) spanning amino acids 208 to 227 of the E1 region,

1bE1 (SEQ ID NO 33) spanning amino acids 192 to 228 of the E1 region,

Env 53 or E1-53 (SEQ ID NO 47) spanning amino acids 313 to 332 of the E1 region,

Env 55 or E1-55 (SEQ ID NO 48) spanning amino acids 325 to 344 of the E1 region.

The present invention also relates to a composition comprising at least one of the following E2 peptides as listed in Table 3:

Env 67 (SEQ ID NO 52) spanning amino acid positions 397 to 416 of the E2 region,

Env 69 (SEQ ID NO 53) spanning amino acid positions 409 to 428 of the E2 region,

Env 23 (SEQ ID NO 66) spanning positions 583 to 602 of the E2 region,

Env 25 (SEQ ID NO 67) spanning positions 595 to 614 of the E2 region,

Env 27 (SEQ ID NO 68) spanning positions 607 to 626 of the E2 region,

Env 17B (SEQ ID NO 63) spanning positions 547 to 566 of the E2 region,

Env 13B (SEQ ID NO 62) spanning positions 523 to 542 of the E2 region.

The present invention also relates to an envelope peptide or protein composition as defined above, for use in a method for immunizing a mammal, preferably humans, against HCV comprising administring a sufficient amount of the composition possibly accompanied by pharmaceutically acceptable adjuvants, to produce an immune response, more particularly a vaccine composition including HCV peptides derived from the E1 and/or the E2 region.

Immunogenic compositions can be prepared according to methods known in the art. The present compositions comprise an immunogenic amount of a polypeptide, e.g. E1 or E2 usually combined with a pharmaceutically acceptable carrier, preferably further comprising an adjuvant. The single envelope proteins E1 or E2 are expected to provide a particularly useful vaccine antigen, since the formation of antibodies to either E1 or E2 may be undesirable, and since the E2 protein is cross-reactive between HCV types and the E1 protein is type-specific. Cocktails including type 1 E2 protein and E1 proteins derived from several genotypes may be particularly advantageous. Immunogenic compositions may be administered to animals to induce production of antibodies, either to provide a source of antibodies or to induce protective immunity in the animal.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to : aluminim hydroxide (alum), N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP) as found in U.S. Patent No. 4,606,918, N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. Any of the 3 components MPL, TDM or CWS may also be used alone or combined 2 by 2. Additionally, adjuvants such as Stimulon (Cambridge Bioscience, Worcester, MA) or SAF-1 (Syntex) may be used. Further, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) may be used for non-human applications and research purposes.

The immunogenic compositions typically will contain pharmaceutically acceptable vehicles, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be included in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect. The E1 and E2 proteins may also be incorporated into Immune Stimulating Complexes together with saponins (ISCOMS).

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the HCV polypeptide, as well as any other of the above mentioned components, as needed. "Immunologically effective amount", means that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment, as defined above. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated

(e.g. nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, the strain of infecting HCV, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The single envelope proteins may also serve as vaccine carriers to present homologous (e.g. T cell epitopes or B cell epitopes from the core, NS2, NS3, NS4 or NS5 regions) or heterologous (non-HCV) haptens, in the same manner as Hepatitis B surface antigen (see European Patent Application 174,444). In this use, envelope proteins provide an immunogenic carrier capable of stimulating an immune response to haptens or antigens conjugated to the aggregate. The antigen may be conjugated either by conventional chemical methods, or may be cloned into the gene encoding E1 and/or E2 at a location corresponding to a hydrophilic region of the protein.

The immunogenic compositions are conventionally administered parenterally, typically by injection, for example, subcutaneously or intramuscularly. Additional formulations suitable for other methods of administration include oral formulations and suppositories. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

The present invention also relates to an E1 or E2 specific antibody raised upon immunization with a peptide or protein composition as defined above by means of a process as defined above, with said antibody being reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

The E1 or E2 specific monoclonal antibodies of the invention can be produced by an hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly from a mouse or rat, immunized against the HCV polypeptides or peptides according to the invention, as defined above on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing the polypeptides which has been initially used for the immunization of the animals.

The antibodies involved in the invention can be labelled by an appropriate label of the enzymatic, fluorescent, or radioactive type.

The monoclonal antibodies according to this preferred embodiment of the invention

may be humanized versions of mouse monoclonal antibodies made by means of recombinant DNA technology, departing from parts of mouse and/or human genomic DNA sequences coding for H and L chains from cDNA clones coding for H and L chains.

Alternatively the monoclonal antibodies according to this preferred embodiment of the invention may be human monoclonal antibodies. These antibodies according to the present embodiment of the invention can also be derived from human peripheral blood lymphocytes of patients infected with HCV, or vaccinated against HCV. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice (for recent review, see Duchosal et al., 1992).

The invention also relates to the use of the proteins or peptides of the invention, for the selection of recombinant antibodies by the process of repertoire cloning (Persson et al., 1991).

Antibodies directed to peptides derived from a certain genotype may be used either for the detection of such HCV genotypes, or as therapeutic agents.

The present invention also relates to the use of a peptide or protein composition as defined above for incorporation into an immunoassay for detecting HCV, present in biological sample liable to contain it, comprising at least the following steps :

(i) contacting the biological sample to be analyzed for the presence of HCV antibodies with any of the peptide or protein compositions as defined above preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex, wherein said peptide can be a biotinylated peptide which is covalently bound to a solid substrate by means of streptavidin or avidin complexes,

(ii) removing unbound components,

(iii) incubating the immune complexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies having conjugated to a detectable label under appropriate conditions,

(iv) detecting the presence of said immunecomplexes visually or by means of densitometry.

Alternatively, the present invention also relates to the incorporation of any of the above-specified E1 or E2 specific monoclonal antibodies in an immunoassay for detecting the presence of E1 or E2 antigen in a biological sample.

Alternatively, the present invention also relates to competition immunoassay formats in which recombinantly produced purified single protein E1 and/or E2 proteins as disclosed above are used in combination with E1 and/or E2 peptides in order to compete for HCV antibodies present in a biological sample possibly containing them.

The present invention also relates to the use of a peptide or protein composition as defined above, for incorporation into a serotyping assay for detecting one or more serological types of HCV present. in a biological sample liable to contain it, more particularly for detecting E1 and/or E2 antigens or antibodies of the different types to be detected combined in one assay format, comprising at least the following steps :

(i) contacting the biological sample to be analyzed for the presence of HCV antibodies or antigens of one ore more serological types, with at least one of the compositions as defined above, an immobilized form under appropriate conditions which allow the formation of an immunecomplex,

(ii) removing unbound components,

(iii) incubating the immunecomplexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies being conjugated to a detectable label under appropriate conditions,

(iv) detecting the presence of said immunecomplexes visually or by means of densitometry and inferring the presence of one or more HCV serological types present from the observed binding pattern.

The present invention also relates to the use of a peptide or protein composition as defined above, for immobilization on a solid substrate and incorporation into a reversed phase hybridization assay, preferably for immobilization as parallel lines onto a solid support such as a membrane strip, for determining the presence or the genotype of HCV according to a method as defined above.

The present invention also relates to a kit for determining the presence of HCV antibodies as defined above present in a biological sample liable to contain them, comprising :

- at least one peptide or protein composition as defined above, preferentially in combination with other polypeptides or peptides from HCV or other types of HCV, with said peptides being preferentially immobilized on a solid substrate, and more preferentially on one and the same membrane strip,
- a buffer or components necessary for producing the buffer enabling binding

reaction between these polypeptides or peptides and the antibodies against HCV present in the biological sample,

- means for detecting the immunecomplexes formed in the preceding binding reaction,
- possibly also including an automated scanning and interpretation device for inferring the HCV genotypes present in the sample from the observed binding pattern.

Alternatively, the present invention also relates to a kit for determining the presence of HCV E1 and/or E2 antigens using HCV E1 and/or E2 specific antibodies according to the present invention.

The immunoassay methods according to the present invention utilize single antigens from the E1 and/or E2 domains that maintain linear and conformational epitopes recognized by antibodies in the sera from individuals infected with HCV. It is within the scope of the invention to use single antigens, dimeric antigens, as well as combinations of single antigens. The HCV E1 and E2 antigens of the present invention may be employed in virtually any assay format that employs a known antigen to detect antibodies. Of course, a format that denatures the HCV conformational epitope should be avoided or adapted. A common feature of all of these assays is that the antigen is contacted with the body component suspected of containing HCV antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will typically be physiologic temperature pH and ionic strenght using an excess of antigen. The incubation of the antigen with the specimen is followed by detection of immune complexes comprised of the antigen.

Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports, or immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the immune complex are also known; examples of which are assays which utilize biotin and avidin or streptavidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a standard or competitive type. In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used

are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidine fluoride (known as Immunlon™), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech Immunlon™ 1 or Immunlon™ 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are know in the art.

In a homogenous format, the test sample is incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of HCV antibodies in the antibody-antigen complexes is directly monitored. This may be accomplished by determining whether labeled anti-xenogeneic (e.g. anti-human) antibodies which recognize an epitope on anti-HCV antibodies will bind due to complex formation. In a competitive format, the amount of HCV antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-HCV antibody (or in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabeled HCV antibodies in the complex may be detected using a conjugate of anti-xenogeneic Ig complexed with a label (e.g.: an enzyme label).

In an immunoprecipitation or agglutination assay format the reaction between the HCV antigens and the antibody forms a network that precipitates from the solution or suspension and forms a visible layer or film of precipitate. If no anti-HCV antibody is present in the test specimen, no visible precipitate is formed.

There currently exist three specific types of particle agglutination (PA) assays. These assays are used for the detection of antibodies to various antigens when coated to a support. One type of this assay is the hemagglutination assay using red blood cells (RBCs) that are sensitized by passively adsorbing antigen (or antibody) to the RBC. The addition of specific antigen antibodies present in the body component, if any, causes the RBCs coated with the purified antigen to agglutinate.

To eliminate potential non-specific reactions in the hemagglutination assay, two

artificial carriers may be used instead of RBC in the PA. The most common of these are latex particles. However, gelatin particles may also be used. The assays utilizing either of these carriers are based on passive agglutination of the particles coated with purified antigens.

The HCV E1 and E2 antigens comprised of conformational epitopes will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the native HCV antigen, control antibody formulations (positive and/or negative), labeled antibody when the assay format requires same and signal generating reagents (e.g. enzyme substrate) if the label does not generate a signal directly. The native HCV antigen may be already bound to a solid matrix or separate with reagents for binding it to the matrix. Instructions (e.g. written, tape, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

Immunoassays that utilize the native HCV antigen are useful in screening blood for the preparation of a supply from which potentially infective HCV is lacking. The method for the preparation of the blood supply comprises the following steps. Reacting a body component, preferably blood or a blood component, from the individual donating blood with native HCV E1 or native HCV E2 antigen to allow an immunological reaction between HCV antibodies, if any, and the HCV antigen. Detecting whether anti-HCV antibody - HCV antigen complexes are formed as a result of the reacting. Blood contributed to the blood supply is from donors that do not exhibit antibodies to the native HCV antigens, E1 or E2.

In cases of a positive reactivity to the HCV antigen, it is preferable to repeat the immunoassay to lessen the possibility of false positives. For example, in the large scale screening of blood for the production of blood products (e.g. blood transfusion, plasma, Factor VIII, immunoglobulin, etc.) "screening" tests are typically formatted to increase sensitivity (to insure no contaminated blood passes) at the expense of specificity; i.e. the false-positive rate is increased. Thus, it is typical to only defer for further testing those donors who are "repeatedly reactive"; i.e. positive in two or more runs of the immunoassay on the donated sample. However, for confirmation of HCV-positivity, the 'confirmation' tests are typically formatted to increase specificity (to insure that no false-positive samples are confirmed) at the expense of sensitivity. Therefore the purification method described in the present invention for E1 and E2 will be very advantageous for including single envelope proteins into diagnostic assays.

The solid phase selected can include polymeric or glass beads, nitrocellulose, microparticles, wells of a reaction tray, test tubes and magnetic beads. The signal

generating compound can include an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound. Examples of enzymes include alkaline phosphatase, horseradish peroxidase and beta-galactosidase. Examples of enhancer compounds include biotin, anti-biotin and avidin. Examples of enhancer compounds binding members include biotin, anti-biotin and avidin. In order to block the effects of rheumatoid factor-like substances, the test sample is subjected to conditions sufficient to block the effect of rheumatoid factor-like substances. These conditions comprise contacting the test sample with a quantity of anti-human IgG to form a mixture, and incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid factor-like substance.

The present invention further contemplates the use of E1 proteins, or parts thereof, more particularly HCV single E1 proteins as defined above, for *in vitro* monitoring or prognosing the response to Interferon treatment of patients suffering from HCV infection comprising:

- incubating a biological sample from a patient receiving interferon therapy with an E1 protein or a suitable part thereof under conditions allowing the formation of an immunological complex,
- removing unbound components,
- calculating the anti-E1 titers present in said sample at the start of and/or during the course of interferon therapy,
- possibly comparing said anti-E1 titers with the anti-E1 titers present during the course of interferon therapy in a sample of a patient which is a long term, sustained responder and of a patient which is a non responder to interferon therapy,
- prognosing the response to interferon treatment of said patient on the basis of the amount anti-E1 titers found in said sample at the start of treatment and/or during the course of treatment.

Patients who show a decrease of 2, 5, 10, or preferably more than 20 times of the in . .. anti-E1 titers could be concluded to be long-term, sustained responders to interferon therapy. It is illustrated in the Examples section, that an anti-E1 assay may be very useful for prognosing long-term response to IFN treatment, or to treatment of Hepatitis C virus disease in general.

More particularly the following E1 peptides as listed in Table 3 were found to be useful for *in vitro* monitoring or prognosing the response to interferon treatment of patients

suffering from HCV infection:

Env 31 or E1-31 (SEQ ID NO 36) spanning amino acids 181 to 200 of the E1 region,

Env 35 or E1-35 (SEQ ID NO 38) spanning amino acids 205 to 224 of the E1 region,

Env 35A or E1-35A (SEQ ID NO 39) spanning amino acids 208 to 227 of the E1 region,

1bE1 (SEQ ID NO 33) spanning amino acids 192 to 228 of the E1 region,

Env 53 or E1-53 (SEQ ID NO 47) spanning amino acids 313 to 332 of the E1 region,

Env 55 or E1-55 (SEQ ID NO 48) spanning amino acids 325 to 344 of the E1 region.

The present invention also relates to a kit for monitoring or prognosing the response to interferon treatment of patients suffering from HCV infection comprising:

- at least one E1 protein or E1 peptide, more particularly an E1 protein or E1 peptide as defined above,
- a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-E1 antibodies present in a biological sample,
- means for detecting the immune complexes formed in the preceding binding reaction,
- possibly also an automated scanning and interpretation device for inferring a decrease of anti-E1 titers during the progression of treatment.

Figure and Table legends

Figure 1 :	Restriction map of plasmid pgpt ATA 18
Figure 2 :	Restriction map of plasmid pgs ATA 18
Figure 3 :	Restriction map of plasmid pMS 66
Figure 4 :	Restriction map of plasmid pv HCV-IIA
Figure 5 :	Anti-E1 levels in non-responders to IFN treatment
Figure 6 :	Anti-E1 levels in responders to IFN treatment
Figure 7 :	Anti-E1 levels in patients with complete response to IFN treatment
Figure 8 :	Anti-E1 levels in incomplete responders to IFN treatment
Figure 9 :	Anti-E2 levels in non-responders to IFN treatment
Figure 10 :	Anti-E2 levels in responders to IFN treatment
Figure 11 :	Anti-E2 levels in incomplete responders to IFN treatment
Figure 12 :	Anti-E2 levels in complete responders to IFN treatment
Figure 13 :	Human anti-E1 reactivity competed with peptides
Figure 14 :	Competition of reactivity of anti-E1 monoclonal antibodies with peptides
Figure 15 :	Anti-E1 (epitope 1) levels in non-responders to IFN treatment
Figure 16 :	Anti-E1 (epitope 1) levels in responders to IFN treatment
Figure 17 :	Anti-E1 (epitope 2) levels in non-responders to IFN treatment
Figure 18 :	Anti-E1 (epitope 2) levels in responders to IFN treatment
Figure 19 :	Competition of reactivity of anti-E2 monoclonal antibodies with peptides
Figure 20:	Human anti-E2 reactivity competed with peptides
Figure 21:	Sequences of the present invention

Table 1 :	Features of the respective clones and primers used for amplification for constructing the different forms of the E1 protein as despected in Example 1.
Table 2	Summary of Anti-E1 tests
Table 3	Synthetic peptides for competition studies

Example 1: Cloning and expression of the hepatitis C virus E1 protein

1. Construction of vaccinia virus recombination vectors

The pgptATA18 vaccinia recombination plasmid is a modified version of pATA18 (Stunnenberg et al, 1988) with an additional insertion containing the *E. coli* xanthine guanine phosphoribosyl transferase gene under the control of the vaccinia virus I3 intermediate promoter (Figure 1). The plasmid pgsATA18 was constructed by inserting an oligonucleotide linker with SEQ ID No. 1, containing stop codons in the three reading frames, into the Pst I and HindIII-cut pATA18 vector. This created an extra PacI restriction site (Figure 2). The HindIII site was not restored.

Oligonucleotide linker with SEQ ID No. 1:

```
5'        G GCATGC AAGCTT AATTAATT        3'
3'    ACGTC CGTACG TTCGAA TTAATTAA TCGA   5'

      PstI  SphI  HindIII  Pac I (HindIII)
```

In order to facilitate rapid and efficient purification by means of Ni^{2+} chelation of engineered histidine stretches to the recombinant proteins, the vaccinia recombination vector pMS66 was designed to express secreted proteins with an additional carboxy-terminal histidine tag. An oligonucleotide linker with SEQ ID No. 2, containing unique sites for 3 restriction enzymes generating blunt ends (Sma I, Stu I and Pml I/Bbr PI) was synthesized in such a way that tlhe carboxy-terminal end of any cDNA could be inserted in frame with a sequence encoding the protease factor Xa cleavage site followed by a nucleotide sequence encoding 6 histidines, 2 stop codons and a Pac I site). This oligonucleotide with SEQ ID No. 2 was introduced between the Xma I and Pst I sites of pgptATA18 (Figure 3).

Oligonucleotide linker with SEQ ID No. 2:

```
5'  [illegible] G GAGGCCTGCACGTGATCGAGGGCAGACACCATCACCACCATCACTAATAGTTAATTAA CTGCA3'
3'      C CTCCGGACGTGCACTAGCTCCCGTCTGTGGTAGTGGTGGTAGTGATTATCAATTAATT G      5'

    XmaI                                                                PstI
```

2. Construction of HCV recombinant plasmids

2.1. Constructs encoding different forms of the E1 protein

Polymerase Chain Reaction (PCR) products were derived from the serum samples by RNA preparation and subsequent reverse-transcription and PCR as described previously (Stuyver et al., 1993b). Table 1 shows the features of the respective clones and the primers used for amplification. The PCR fragments were cloned into the SmaI-cut pSP72 (Promega) plasmids. The following clones were selected for insertion into vaccinia reombination vectors: HCCI9A (SEQ ID NO. 3), HCCI10A (SEQ ID NO. 4), HCCI11A (SEQ ID NO. 5), HCCI12A (SEQ ID NO. 6), HCCI13A (SEQ ID NO. 7), and HCCI17A (SEQ ID NO. 8) as despected in Figure 21. cDNA fragments containing the E1-coding regions were cleaved by EcoRI and HindIII restriction from the respective pSP72 plasmids and inserted into the EcoRI/HindIII-cut pgptATA-18 vaccinia recombination vector (described in example 1), downstream of the 11K vaccinia virus late promoter. The respective plasmids were designated pvHCV-9A, pvHCV-10A, pvHCV-11A, pvHCV-12A, pvHCV-13A and pvHCV-17A, of which pvHCV-11A is shown in Figure 4.

2.2. Hydrophobic region E1 deletion mutants

Clone HCCI37, containing a deletion of codons Asp264 to Val287 (nucleotides 790 to 861, region encoding hydrophobic domain I) was generated as follows: 2 PCR fragments were generated from clone HCCI10A with primer sets HCPr52 (SEQ ID NO. 10)/HCPr107 (SEQ ID NO. 13) and HCPr108 (SEQ ID NO. 14)/HCPR54 (SEQ ID NO. 12). The two PCR fragments were purified from agarose gel after electrophoresis and 1 ng of each fragment was used together as template for PCR by means of primers HCPr52 (SEQ ID NO. 9) and HCPr54 (SEQ ID NO. 11). The resulting fragment was cloned into the Sma I-cut pSP72 vector and clones containing the deletion were readily identified because of the deletion of 24 codons (72 base pairs). Plasmid pSP72HCCI37 containing cone HCCI37 (SEQ ID 15) was selected. A recombinant vaccinia plasmid containing the full-length E1 cDNA lacking hydrophobic domain I was constructed by inserting the HCV sequence surrounding the deletion (fragment cleaved by Xma I and BamH I from the vector pSP72-HCCI37) into the Xma I-Bam H I sites of the vaccinia plasmid pvHCV-10A. The resulting plasmid was named pvHCV-37. After confirmatory sequencing, the amino-terminal region

containing the internal deletion was isolated from this vector pvHCV-37 (cleavage by EcoR I and BstE II) and reinserted into the Eco RI and Bst EII-cut pvHCV-11A plasmid. This construct was expected to express an E1 protein with both hydrophobic domains deleted and was named pvHCV-38. The E1-coding region of clone HCCI38 is represented by SEQ ID NO. 16.

As the hydrophylic region at the E1 carboxyterminus (theoretically extending to around amino acids 337 to 340) was not completely included in construct pvHCV-38, a larger E1 region lacking hydrophobic domain I was isolated from the pvHCV-37 plasmid by EcoR I/Bam HI cleavage and cloned into an EcoRI/BamHI-cut pgsATA-18 vector. The resulting plasmid was named pvHCV-39 and contained clone HCCI39 (SEQ ID NO. 17). The same fragment was cleaved from the pvHCV-37 vector by BamH I (of which the sticky ends were filled with Klenow DNA Polymerase I (Boehringer) and subsequently by EcoR I (5' cohesive end). This sequence was inserted into the EcoRI and Bbr PI-cut vector pMS-66. This resulted in clone HCCI40 (SEQ ID NO. 18) in plasmid pvHCV-40.

2.3 E1 of other genotypes

Clone HCCI62 (SEQ ID NO. 19) was derived from a type 3a-infected patient with chronic hepatitis C (serum BR36, clone BR36-9-13, SEQ ID NO. 19 in EPA 93 401 099.2, and see also Stuyver et al. 1993a) and HCCI63 (SEQ ID NO. 20) was derived from a type 5a-infected child with post-transfusion hepatitis (serum BE95, clone PC-4-1, SEQ ID NO. 45 in European patent application 93 401 099.2.).

2.4 E2 constructs

The HCV E2 PCR fragment 22 was obtained from serum BE11 (genotype 1b) by means of primers HCPr109 (SEQ ID NO. 21) and HCPr72 (SEQ ID NO. 22) using techniques of RNA preparation and reverse-transcription and PCR, as described in Stuyver et al., 1993b, and the fragment was cloned into the Sma I-cut pSP72 vector. Clone HCCI22A (SEQ ID NO. 23) was cut with NcoI/AlwNI or by BamHI/AlwNI and the sticky ends of the fragments were blunted (NcoI and BamHI sites with Klenow DNA Polymerase I (Boehringer), and AlwNI with T4 DNA polymerase (Boehringer)). The BamHI/AlwNI cDNA fragment was then inserted into the vaccinia pgsATA-18 vector that had been linearized by EcoR I and Hind III cleavage and of which the cohesive ends had been filled with

Klenow DNA Polymerase (Boehringer). The resulting plasmid was named pvHCV-41 and encoded the E2 region from amino acids Met 347 to Q673, including 37 amino acids from Met 347 to Gly383 of the E1 protein that can serve as signal sequence. The same HCV cDNA was inserted into the EcoR I and Bbr PI-cut vector pMS66, that had subsequently been blunt ended with Klenow DNA Polymerase. The resulting plasmid was named pvHCV-42 and also encoded amino acids 347 to 683. The NcoI/AlwNI fragment was inserted in a similar way into the same sites of pgsATA-18 (pvHCV-43) or pMS-66 vaccinia vectors (pvHCV-44). pvHCV-43 and pvHCV-44 encoded amino acids 364 to 673 of the HCV polyprotein, of which amino acids 364 to 383 were derived from the natural E1 carboxyterminal region of the E1 protein and amino acids 384 to 673 of the E2 protein.

2.5. Generation of recombinant HCV-vaccinia viruses

Rabbit kidney RK_{13} cells (ATCC CCL 37), human ostesarcoma 143B thymidine kinase minus (TK^-) (ATCC CRL 8303), HeLa (ATCC CCL 2), and Hep G2 (ATCC HB 8065) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md, USA). The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % foetal calf serum, and with Earle's salts (EMEM) for RK_{13} and 143 B (TK-), and with glucose (4 g/l) for Hep G2. The vaccinia virus WR strain (Western Reserve, ATTC VR119) was routinely propagated in either 143B or RK_{13} cells, as described previously (Panicali & Paoletti, 1982; Piccini *et al.*, 1987; Mackett *et al.*, 1982, 1984, and 1986). A confluent monolayer of 143B cells was infected with wild type vaccinia virus at a multiplicity of infection (m.o.i.) of 0.1 (= 0.1 plaque forming unit (PFU) per cell). Two hours later, the vaccinia recombination plasmid was transduced into the infected cells in the form of a calcium phosphate coprecipitate containing 500 ng of the plasmid DNA to allow homologous recombination (Graham & van der Eb, 1973; Mackett *et al.*, 1985). Recombinant viruses expressing the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (gpt) protein were selected on rabbit kidney RK_{13} cells incubated in selection medium (EMEM containing 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine, and 15 μg/ml hypoxanthine; Falkner and Moss, 1988; Janknecht et al, 1991). Single recombinant viruses were purified on fresh monolayers of RK_{13} cells under a 0.9% agarose overlay in selection medium. Thymidine kinase deficient (TK^-) recombinant viruses were selected and then plaque purified on fresh monolayers of human 143B cells (TK-) in the presence of 25 μg/ml 5-bromo-2'-deoxyuridine. Stocks of purified recombinant HCV-

vaccinia viruses were prepared by infecting either human 143 B or rabbit RK_{13} cells at an m.o.i. of 0.05 (Mackett et al, 1988). The insertion of the HCV cDNA fragment in the recombinant vaccinia viruses was confirmed on an aliquot (50 μl) of the cell lysate after the MPA selection by means of PCR with the primers used to clone the respective HCV fragments (see Table 1). The recombinant vaccinia-HCV viruses were named according to the vaccinia recombination plasmid number, e.g. the recombinant vaccinia virus vvHCV-10A was derived from recombining the wild type WR strain with the pvHCV-10A plasmid.

Example 3: infection of cells with recombinant vaccinia viruses

A confluent monolayer of RK_{13} cells was infected at a m.o.i. of 3 with the recombinant HCV-vaccinia viruses as describe din example 2 . For infection, the cell monolayer was washed twice with phosphate-buffered saline pH 7.4 (PBS) and the recombinant vaccinia virus stock was diluted in MEM medium. Two hundred μl of the virus solution was added per 10^6 cells such that the m.o.i. was 3, and incubated for 45 min at 24°C. The virus solution was aspirated and 2 ml of complete growth medium (see example 2) was added per 10^6 cells. The cells were incubated for 24 hr at 37°C during which expression of the HCV proteins took place.

Example 4: Analysis of recombinant proteins by means of western blotting

The infected cells were washed two times with PBS, directly lysed with lysis buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 1 ug/ml aprotinin (Sigma, Bornem, Belgium)) or detached from the flasks by incubation in 40 mM Tris.HCL pH 7.5 10 mM EDTA/ 150 mM NaCl for 5 min, and collected by centrifugation (5 min at 1000g: The cell pellet was then resuspended in 200 μl lysis buffer (50 mM Tris.HCL pH 8.0, 2 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, aprotinin, 1% Triton X-100) per 10^6 cells.

The cell lysates were cleared for 5 min at 14,000 rpm in an Eppendorf centrifuge to remove the insoluble debris. Proteins of 20 μl lysate were separated by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then electro-transferred from the gel to a nitrocellulose sheet (Amersham) using a Hoefer HSI transfer unit cooled to 4°C for 2 hr at 100 V constant voltage, in transfer buffer 25 mM Tris.HCl pH 8.0, 192 mM glycine, 20% (v/v) methanol). Nitrocellulose filters were blocked with Blotto (5 % (w v) fat-free instant milk powder in PBS; Johnson

et al., 1981) and incubated with primary antibodies diluted in Blotto/0.1 % Tween 20. Usually, a human negative control serum or serum of a patient infected with HCV were 200 times diluted and preincubated for 1 hour at room temperature with 200 times diluted wild type vaccinia virus-infected cell lysate in order to decrease the non-specific binding. After washing with Blotto/0.1% Tween 20, the nitrocellulose filters were incubated with alkaline phosphatase substrate solution diluted in Blotto/0.1 % Tween 20. After washing with 0.1% Tween 20 in PBS, the filters were incubated with alkaline phosphatase substrate solution (100 mM Tris.HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0,38 μg/ml nitroblue tetrazolium, 0.165 μg/ml 5-bromo-4-chloro-3-indolylphosphate). All steps, except the electrotransfer, were performed at room temperature.

Example 5: Purification of recombinant E1 protein

1. Lysis

Infected RK13 cells were washed 2 times with phosphate-buffered saline (PBS) and detached from the culture recipients by incubation in PBS containing 10 mM EDTA. The detached cells were washed twice with PBS and 1 ml of lysis buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 1 ug/ml aprotinin (Sigma, Bornem, Belgium) containing 2 mM biotinylated N-ethylmaleimide (biotin-NEM) (Sigma) was added per 10 cells at 4°C. This lysate was homogenized with a type B douncer and left at room temperature for 0.5 hours. Another 5 volumes of lysis buffer containing 10 mM N-ethylmaleimide (NEM, Aldrich, Bornem, Belgium) was added to the primary lysate and the mixture was left at room temperature for 15 min. Insoluble cell debris was cleared from the solution by centrifugation in a Beckman JA-14 rotor at 14,000 rpm (30100 g at r_{max}) for 1 hour at 4°C.

2. Lectin Chromatography

The cleared cell lysate was loaded at a rate of 1ml/min on a 0.8 by 10 cm Lentil-lectin Sepharose 4B column (Pharmacia) that had been equilibrated with 5 column volumes of lysis buffer at a rate of 1ml/min. The lentil-lectin column was washed with 5 to 10 column volumes of buffer 1 (0.1M potassium phosphate pH 7.3, 500 mM KCl, 5% glycerol, 1 mM 6-NH_2-hexanoic acid, 1 mM $MgCl_2$, and 1% DecylPEG (KWANT, Bedum,

The Netherlands). In some experiments,the column was subsequently washed with 10 column volumes of buffer 1 containing 0.5% Empigen-BB (Calbiochem, San Diego, CA, USA) instead of 1% DecylPEG. The bound material was eluted by applying elution buffer (10 mM potassium phosphate pH 7.3, 5% glycerol, 1 mM hexanoic acid, 1mM $MgCl_2$, 0.5% Empigen-BB, and 0.5 M α-methyl-mannopyranoside. The eluted material was fractionated and fractions were screened for the presence of E1 protein by means of ELISA as described in example 6.

3. Concentration and partial reduction

The E1-positive fractions were pooled and concentrated on a Centricon 30 kDa (Amicon) by centrifugation for 3 hours at 5,000 rpm in a Beckman JA-20 rotor at 4°C. In some experiments the E1-positive fractions were pooled and concentrated by nitrogen evaporation. An equivalent of 3.10^8 cells was concentrated to approximately 200 μl. For partial reduction, 30% Empigen-BB (Calbiochem, San Diego, CA, USA) was added to this 200 μl to a final concentration of 3.5 %, and 1M DTT in H_2O was subsequently added to a final concentration of 7.5 mM and incubated for 30 min at 37 °C. NEM (1M in dimethylsulphoxide) was subsequently added to a final concentration of 50 mM and left to react for another 30 min at 37°C to block the free sulphydryl groups.

4. Gel filtration chromatography

- Superdex-200 HR 10/20 column (Pharmacia) was equilibrated with 3 column volumes PBS/3% Empigen-BB. The reduced and mixture was injected in a 500 μl sample loop of the Smart System (Pharmacia) and PBS/3% Empigen-BB buffer was added for gelfiltration. Fractions of 250 μl were collected from V_0 to V_t. The fractions were screened for the presence of E1 protein as described in example 6. The presence of purified E1 protein was further confirmed by means of western blotting as described in example 4. The dimeric E1 protein appeared to be non-aggregated and free of contaminants. The NH_2 terminus of the protein was determined and appeared to contain a tyrosine as first residue.

Example 6: ELISA for the detection of anti-E1 antibodies or for the detection of E1 proteins

- mab to capture E1
- direct coating

Maxisorb microwell plates (Nunc, Roskilde, Denmark) were coated with 1 volume (e.g. 50 μl or 100 μl or 200 μl) per well of a 5 μg/ml solution of Strepatavidin (Boehringer Mannheim) in PBS for 16 hours at 4 °C or for 1 hour at 37 °C. Alternatively, the wells were coated with 1 volume of 5 μg/ml of Galanthus nivalis agglutinin (GNA) in 50 mM sodium carbonate buffer pH 9.6 for 16 hours at 4 °C or for 1 hour at 37 °C. In the case of coating with GNA, the plates were washed 2 times with 400 μl of Washing Solution of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium). Unbound coating surfaces were blocked with 1.5 to 2 volumes of blocking solution (0.1 % casein and 0.1 % NaN_3 in PBS) for 1 hour at 37 °C or for 16 hours at 4 °C. Blocking solution was aspirated. Purified E1 was diluted to 100 ng/ml (concentration measured at A = 280 nm) or column fractions to be screened for E1 (see example 5), or E1 in non-purified cell lysates (example 5.1.) were diluted 20 times in blocking solution, and 1 volume of the E1 solution was added to each well and incubated for 1 hour at 37 °C on the Streptavidin- or GNA-coated plates. The microwells were washed 3 times with 1 volume of Washing Solution of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium). Serum samples were diluted 20 times or monoclonal anti-E1 antibodies were diluted to a concentration of 20 ng/ml in Sample Diluent of the Innotest HCV Ab III kit and 1 volume of the solution was left to react with the E1 protein for 1 hour at 37 °C. The microwells were washed 5 times with 400 μl of Washing Solution of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium). The bound antibodies were detected by incubating each well for 1 hour at 37 °C with a goat anti-human or anti-mouse IgG, peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) diluted 1/80,000 in 1 volume of Conjugate Diluent of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium), and color development was obtained by addition of substrate of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium) diluted 100 times in 1 volume of Substrate Solution of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium) for 30 min at 24 °C after washing of the plates 3 times with 400 μl of Washing Solution of the Innotest HCV Ab III kit (Innogenetics, Zwijndrecht, Belgium).

Example 7: Follow up of patient groups with different clinical profiles

7.1. Monitoring of anti-E1 and anti-E2 antibodies

The current hepatitis C virus (HCV) diagnostic assays have been developed for screening and confirmation of the presence of HCV antibodies. Such assays do not seem to provide information useful for monitoring of treatment or for prognosis of the outcome of disease. However, as is the case for hepatitis B, detection and quantification of anti-envelope antibodies may prove more useful in a clinical setting. To investigate the possibility of the use of anti-E1 antibody titer and anti-E2 antibody titer as prognostic markers for outcome of hepatitis C disease, a series of IFN-α treated patients with long-term sustained response (defined as patients with normal transaminase levels and negative HCV-RNA test (PCR in the 5' non-coding region) in the blood for a period of at least 1 year after treatment) was compared with patients showing no response or showing biochemical response with relapse at the end of treatment.

A group of 8 IFN-α treated patients with long-term sustained response (LTR, follow up 1 to 3.5 years, 3 type 3a and 5 type 1b) was compared with 9 patients showing non-complete responses to treatment (NR, follow up 1 to 4 years, 6 type 1b and 3 type 3a). Type 1b (vvHCV-39, see example 2.5.) and 3a E1 (vvHCV-62, see example 2.5.) proteins were expressed by the vaccinia virus system (see examples 3 and 4) and purified to homogeneity (example 5). The samples derived from patients infected with a type 1b hepatitis C virus were tested for reactivity with purified type 1b E1 protein, while samples of a type 3a infection were tested for reactivity of anti-type 3a E1 antibodies in an ELISA as desribed in example 6. The genotypes of hepatitis C viruses infecting the different patients were determined by means of the Inno-LiPA genotyping assay (Innogenetics, Zwijnaarde, Belgium). Figure 5 shows the anti-E1 signal-to-noise ratios of these patients followed during the course of interferon treatment and during the follow-up period after treatment. LTR cases consistently showed rapidly declining anti-E1 levels (with complete negativation in 3 cases), while anti-E1 levels of NR cases remained approximately constant. Some of the obtained anti-E1 data are shown in Table 2 as average S/N ratios ± SD (mean anti-E1 titer). The anti-E1 titer could be deduced from the signal to noise ratio as shown in Figures 5, 6, 7, and 8.

Already at the finish of treatment, marked differences could be observed between the two groups. Anti-E1 antibody titers had decreased 6.9 times in LTR but only 1.5 times

in NR. At the end of follow up, the anti-E1 titers had declined by a factor of 22.5 in the patients with sustained response and even slightly increased in NR. Therefore, based on these data, decrease of anti-E1 antibody levels during monitoring of IFN-α therapy correlates with long-term, sustained response to treatment. The anti-E1 assay may be very useful for prognosis of long-term response to IFN treatment, or to treatment of the hepatitis C disease in general.

This finding was not expected. On the contrary, the inventors had expected the anti-E1 antibody levels to increase during the course of IFN treatment in patients with long term response. As is the case for hepatitis B, the virus is cleared as a consequence of the seroconversion for anti-HBsAg antibodies. Also in many other virus infections, the virus is eliminated when anti-envelope antibodies are raised. However, in the experiments of the present invention, anti-E1 antibodies clearly decreased in patients with a long -term response to treatment, while the antibody-level remained approximately at the same level in non-responding patients. Although the outcome of these experiments was not expected, this non-obvious finding may be very important and useful for clincial diagnosis of HCV infections. As shown in Figures 9, 10, 11, and 12, anti-E2 levels behaved very different in the same patients studied and no obvious decline in titers was observed as for anti-E1 antibodies.

As can be deduced from Table 2, the anti-E1 titers were upon an average at least 2 times higher at the start of treatment in long term responders compaired with incomplete responders to treatment. Therefore, measuring the titer of anti-E1 antibodies at the start of treatment, or monitoring the patient during the course of infection and measuring the anti-E1 titer, may become a useful marker for clinical diagnosis of hepatitis C. Furthermore, the use of more defined regions of the E1 or E2 proteins may become desirable, as shown in example 7.2.

7.2 Monitoring of antibodies of defined regions of the E1 protein

Several 20-mer peptides (Table 3) that overlapped each other by 8 amino acids, were synthesized according to method described previously (EP-A-0 489 968) based on the HCV-J1 sequence (Okamoto et al., 1990). None of these, except peptide env35 (also referred to as E1-35), was able to detect antibodies in sera of approximately 200 HCV cases. Only 2 sera reacted slightly with the env35 peptide. However, by means of the anti-E1 ELISA as described in example 6, it was possible to discover additional epitopes as

follows. The anti-E1 ELISA as described in example 6 was modified by mixing 50 µg/ml of E1 peptide with the 1/20 diluted human serum in sample diluent. Figure 13 shows the results of reactivity of human sera to the recombinant E1 (expressed from vvHCV-40) protein, in the presence of single or of a mixture of E1 peptides. While only 2% of the sera could be detected by means of E1 peptides coated on strips in a Line Immunoassay format, over half of the sera contained anti-E1 antibodies which could be competed by means of the same peptides, when tested on the recombinant E1 protein. Some of the murine monoclonal antibodies obtained from Balb/C mice after injection with purified E1 protein were subsequently competed for reactivity to E1 with the single peptides (Figure 14). Clearly, the region of env53 contained the predominant epitope, as the addition of env53 could substantially compete reactivity of several sera with E1, and antibodies to the env31 region were also detected. This finding was surprising, since the env53 and env31 peptides had not shown any reactivity when coated directly to the solid phase.

Therefore peptides were synthesized using technology described by applicant previously (in WO 93/18054). The following peptides were synthesized:

peptide env35A-biotin

NH_2-SNSSEAADMIMHTPGCV-biotin (SEQ ID NO 31)

spanning amino acids 208 to 227 of the HCV polyprotein in the E1 region

peptide biotin-env53 ('epitope 1')

biotin-GG-ITGHRMAWDMMMNWSPTTAL-$_{COOH}$ (SEQ ID NO. 32)

spanning amino acids to 313 of 332 of the HCV polyprotein in the E1 region

peptide 1bE1 ('epitope 2')

H_2N-YEVRNVSGIYHVTNDCSNSSIVYEAADMIMHTPGCGK -G-biotin (SEQ ID NO. 33)

spanning amino acids 192 to 228 of the HCV polyprotein in the E1 region

and compared with the reactivities of peptides E1a-BB (biotin-GG-TP[illegible]RDGKLPATQLRRHIDLL, SEQ ID NO. 34) and E1b-BB (biotin-GG-TP[illegible]RDASVPTTTIRRHVDLL, SEQ ID NO. 35) which are derived from the same region of sequences of genotype 1a and 1b respectively and which have been described at the IXth international virology meeting in Glasgow, 1993 ('epitope 3'). Reactivity of a panel of HCV sera was tested on epitopes 1, 2, and 3 and epitope 2 was also compared with

env35A (of 47 HCV-positive sera, 8 were positive on epitope 2 and none reacted with env35A). Reactivity towards epitopes 1, 2 and 3 was tested directly to the biotinylated peptides (50 μg/ml) bound to streptavidin-coated plates as described in example 6. Clearly, epitopes 1 and 2 were most reactive while epitopes 3 and env35A-biotin were much less reactive. The same series of patients that had been monitored for their reactivity towards the complete E1 protein (example 7.1.) was tested for reactivity towards epitopes 1, 2, and 3. Little reactivity was seen to epitope 3, while as shown in Figures 15, 16, 17, and 18, epitopes 1 and 2 reacted with the majority of sera. However, antibodies to the most reactive epitope (epitope 1) did not seem to predict remission of disease, while the anti-1bE1 antibodies (epitope 2) were present almost exclusively in long term responders at the start of IFN treatment. Therefore, anti-1bE1 (epitope 2) antibodies and anti-env53 (epitope 1) antibodies could be shown to be useful markers for prognosis of hepatitis C disease. The env53 epitope may be advantageously used for the detection of cross-reactive antibodies (antibodies that cross-react between major genotypes) and antibodies to the env53 region may be ver useful for universal E1 antigen detection in serum or liver tissue. Monoclonal antibodies that recognized the env53 region were reacted with a random epitope library in plasmids. In 4 clones that proved reacted upon immunoscreening with the monoclonal antibody 5E1A10, the sequence -GWD- was present. Because of its analogy with the universal HCV sequence present in all HCV variants in the env53 region, the sequence AWD is thought to contain the core sequence of the env53 cross-reactive murine epitope. The env31 clearly also contains an epitope in the amino terminal sequence -YQVRNSTGL- and may be useful for diagnosis. Env31 or E1-31 as shown in Table 3, is a part of the peptide 1bE1.

Anti-E2 antibodies clearly followed a different pattern than the anti-E1 antibodies, especially in patients with a long-term response to treatment. Therefore, it is clear that the decrease in anti-envelope antibodies could not be measured as efficiently with an assay employing a recombinant E1/E2 protein as with a single anti-E1 or anti-E2 protein. The anti-E2 response would clearly blur the anti-E1 response in an assay measuring both kinds of antibodies at the same time. Therefore, the ability to test anti-envelope antibodies to the single E1 and E2 proteins, was shown to be useful.

7.3 Monitoring of antibodies of defined regions of the E2 protein

Of the 24 anti-E2 Mabs only three could be competed for reactivity to recombinant

E2 by peptides, two of which reacted with the HVRI region (peptides E2-67 and E2-69, designated as epitope A) and one which recognized an epitope competed by peptide E2-13B (epitope C). The majority of murine antibodies recognized conformational anti-E2 epitopes (Figure 19). A human response to HVRI (epitope A), and to a lesser extent HVRII (epitope B) and a third linear epitope region (competed by peptides E2-25 or E2-27, designated epitope D) could also frequently be observed, but the majority of sera reacted with conformational epitopes (Figure 20).

Table 1

Plasmid	Construction	Length (nt/aa)
pvHCV-13A	EcoR I - Hind III	472/157
pvHCV-12A	EcoR I - Hind III	472/158
pvHCV-9A	EcoR I - Hind III	631/211
pvHCV-11A	EcoR I - Hind III	625/207
pvHCV-17A	EcoR I - Hind III	625/208
pvHCV-10A	EcoR I - Hind III	783/262
pvHCV-18A	Acc I (KI) - EcoR I (KI)	403/130
pvHCV-34	Acc I (KI) - Fsp I	595/197
pvHCV-33	Acc I (KI)	1150/380
pvHCV-35	EcoR I - BamH I (KI)	1032/352
pvHCV-36	EcoR I - Nco I (KI)	1106/376
pvHCV-37	Xma I - BamH I	711/239
pvHCV-38	EcoR I - BstE II	553/183
pvHCV-39	EcoR I - BamH I	960/313
pvHCV-40	EcoR I - BamH I (KI)	960/323
pvHCV-41	BamH I (KI)-AlwN I (T4)	1005/331
pvHCV-42	BamH I (KI)-AlwN I (T4)	1005/341
pvHCV-43	Nco I (KI) - AlwN I (T4)	932/314
pvHCV-44	Nco I (KI) - AlwN I (T4)	932/321

pvHCV-19	Nco I (Kl) - Hind III	658/319
pvHCV-59	Acc I (Kl) - Fsp I	595/199
pvHCV-62	EcoR I - Hind III	625/207
pvHCV-63	EcoR I - Hind III	625/207
pvHCV-64	BamH I - Hind III	
pvHCV-65	BamH I - Hind III	
pvHCV-66	BamH I - Hind III	

nt, nucleotide, aa, aminoacid.

Table 2

SUMMARY OF ANTI-E1 TESTS

S/N ± SD (mean anti-E1 titer)

	Start of treatment	End of treatment	Follow-up
LTR	6.94 ± 2.29 (1:3946)	4.48 ± 2.69 (1:568)	2.99 ± 2.69 (1:175)
NR	5.77 ± 3.77 (1:1607)	5.29 ± 3.99 (1:1060)	6.08 ± 3.73 (1:1978)

LTR: Long-term, sustained response for more than 1 year

NR: No response, response with relapse, or partial response

TABLE 3
Synthetic peptides for competition studies

PROTEIN	PEPTIDE	AMINO ACID SEQUENCE	POSITION	SEQ ID NO
E1	E1-31	LLSCLTVPASAYQVRNSTGL	181-200	36
	E1-33	QVRNSTGLYHVTNDCPNSSI	193-212	37
	E1-35	NDCPNSSIVYEAHDAILHTP	205-224	38
	E1-35A	SNSSIVYEAADMIMHTPGCV	208-227	39
	E1-37	HDAILHTPGCVPCVREGNVS	217-236	40
	E1-39	CVREGNVSRCWVAMTPTVAT	229-248	41
	E1-41	AMTPTVATRDGKLPATQLRR	241-260	42
	E1-43	LPATQLRRHIDLLVGSATLC	253-272	43
	E1-45	LVGSATLCSALYVGDLCGSV	265-284	44
	E1-49	QLFTFSPRRHWTTQGCNCSI	289-308	45
	E1-51	TQGCNCSIYPGHITGHRMAW	301-320	[illegible]
	E1-53	ITGHRMAWDMMMNWSPTAAL	313-332	47
	E1-55	NWSPTAALVMAQLLRIPQAI	325-344	48
	E1-57	LLRIPQAILDMIAGAHWGVL	337-356	49
	E1-59	AGAHWGVLAGIAYFSMVGNM	349-368	50
	E1-63	VVLLLFAGVDAETIVSGGQA	373-392	51
E2	E2-67	SGLVSLFTPGAKQNIQLINT	397-416	52
	E2-69	QNIQLINTNGSWHINSTALN	409-428	53
	E2-$3B	LNCNESLNTGWWLAGLIYQHK	427-446	54
	E2-$1B	AGLIYQHKFNSSGCPERLAS	439-458	55
	E2-1B	GCPERLASCRPLTDFDQGWG	451-470	56
	E2-3B	TDFDQGWGPISYANGSGPDQ	463-482	57
	E2-5B	ANGSGPDQRPYCWHYPPKPC	475-494	58
	E2-7B	WHYPPKPCGIVPAKSVCGPV	487-506	[illegible]
	E2-9B	AKSVCGPVYCFTPSPVVVGT	499-518	[illegible]
	E2-11B	PSPVVVGTTDRSGAPTYSWG	511-530	61
	E2-13B	GAPTYSWGENDTDVFVLNNT	523-542	62
	E2-17B	GNWFGCTWMNSTGFTKVCGA	547-566	63
	E2-19B	GFTKVCGAPPVCIGGAGNNT	559-578	64
	E2-21	IGGAGNNTLHCPTDCFRKHP	571-590	65
	E2-23	TDCFRKHPDATYSRCGSGPW	583-602	66
	E2-25	SRCGSGPWITPRCLVDYPYR	595-614	67
	E2-27	CLVDYPYRLWHYPCTINYTI	607-626	68
	E2-29	PCTINYTIFKIRMYVGGVEH	619-638	69
	E2-31	MYVGGVEHRLEAACNWTPGE	631-650	70
	E2-33	ACNWTPGERCDLEDRDRSEL	643-662	71
	E2-35	EDRDRSELSPLLLTTTQWQV	655-674	72

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Claims

1. Composition comprising essentially purified HCV single envelope proteins selected from the group consisting of E1 and E2, characterized as being isolated in the presence of a means for cleaving disulphide bonds and preferably also in the presence of a means for blocking disulphide bond reformation.

2. Recombinant vector comprising a vector sequence, an appropriate (prokaryotic,) eukaryotic or viral promoter sequence followed by a nucleotide sequence allowing the expression of an E1 or E2 protein, with said segment of HCV encoding the desired E1 and/or E2 proteins possibly being attached to a signal sequence, preferably a signal sequence appearing in the HCV genome before the respective start points of the E1 and/or E2 proteins, and with said segment of HCV encoding the desired E1 and/or E2 proteins possibly including a deletion of the hybdrophobic domain(s) of the respective E1 and/or E2 proteins.

3. Recombinant vector according to claim 2, comprising any of the sequences as represented in SEQ ID NO 3 to 30.

4. A host cell transformed with a recombinant vector according to any of claims 2 to 3, wherein said vector comprises a nucleotide sequence encoding HCV E1 and/or E2 protein as defined in any of claims 2 to 3 in addition to a regulatory sequence operable in said host cell and capable of regulating expression of said HCV E1 and/or E2 protein.

5. Method for producing and purifying HCV single envelope proteins selected from the group consisting of E1 and E2, comprising the essential step of incubation with a disulphide bond cleaving agent, and preferably also incubation with an agent preventing disulphide bond (re)formation.

6. Method according to claim 5, further characterized as comprising the following steps:

- growing a lower or higher eukaryote host cell as defined in claim 4 transformed with a recombinant vector according to any of claims 2 to 3 in a suitable culture

medium,

- causing expression of said vector sequence as defined in any of claims 2 to 3 under suitable conditions, and,
- recovering said recombinant HCV protein from said cell culture by lysing said cells, preferably in the presence of a SH group blocking agent, such as N-ethylmaleimide (NEM),
- recovering said HCV single envelope protein by affinity purification by means of lectin-chromatography or immunoaffinity chromatography using anti-E1 and/or anti-E2 specific monoclonal antibodies, with said lectin being preferably lentil-lectin, followed by,
- incubation of the eluate of the previous step with a disulphide bond cleavage means, such as DTT, preferably also in the presence of an SH group blocking agent, such as NEM or Biotin-NEM, and,
- isolating the HCV single E1 and/or E2 proteins by means of gelfiltration or Ni-IMAC.

7. A composition comprising at least one of the following E1 and/or E2 peptides:

Env 31 or E1-31 (SEQ ID NO 36) spanning amino acids 181 to 200 of the E1 region,

Env 35 or E1-35 (SEQ ID NO 38) spanning amino acids 205 to 224 of the E1 region,

Env 35A or E1-35A (SEQ ID NO 39) spanning amino acids 208 to 227 of the E1 region,

1bE1 (SEQ ID NO 33) spanning amino acids 192 to 228 of the E1 region,

Env 53 or E1-53 (SEQ ID NO 47) spanning amino acids 313 to 332 of the E1 region,

Env 55 or E1-55 (SEQ ID NO 48) spanning amino acids 325 to 344 of the E1 region,

Env 67 (SEQ ID NO 52) spanning amino acid positions 397 to 416 of the E2 region,

Env 69 (SEQ ID NO 53) spanning amino acid positions 409 to 428 of the E2 region,

Env 23 (SEQ ID NO 66) spanning positions 583 to 602 of the E2 region,

Env 25 (SEQ ID NO 67) spanning positions 595 to 614 of the E2 region,

Env 27 (SEQ ID NO 68) spanning positions 607 to 626 of the E2 region,

Env 17B (SEQ ID NO 63) spanning positions 547 to 566 of the E2 region,

Env 13B (SEQ ID NO 62) spanning positions 523 to 542 of the E2 region.

8. A composition according to any of claims 1 or 7, for use in a method for immunizing a mammal, preferably a human, against HCV, comprising administrating an effective amount of said composition possibly accompanied by pharmaceutically acceptable adjuvants, to produce an immune response.

9. An E1 and/or E2 specific monoclonal antibody raised upon immunization with any of the protein or peptide compositions according to any of claims 1 or 7.

10. Method for in vitro diagnosis of HCV present in a biological sample, comprising at least the following steps:

(i) contacting said biological sample with a composition according to any of claims 1 or 7, preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex,

(ii) removing unbound components,

(iii) incubating the immune complexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies being conjugated to a detectable label under appropriate conditions,

(iv) detecting the presence of said immunecomplexes visually or by means of densitometry.

11. Kit for determining the presence of HCV antibodies present in a biological sample, comprising:

- at least one peptide or protein composition according to any of claims 1 or 7, preferably in an immobilized form on a solid substrate,
- a buffer or components necessary for producing the buffer enabling binding reaction between these proteins or peptides and the antibodies against HCV present in said biological sample,
- a means for detecting the immune complexes formed in the preceding binding reaction.

12. Use of E1 proteins, or parts thereof, more particularly HCV single E1 proteins according to claim 1 or E1 peptides according to claim 7, for *in vitro* monitoring or prognosing the response to Interferon treatment of patients suffering from HCV infection comprising:

- incubating a biological sample from a patient receiving interferon therapy with an E1 protein or a suitable part thereof under conditions allowing the formation of an immunological complex,
- removing unbound components,
- calculating the anti-E1 titers present in said sample at the start of and during the course of interferon therapy,
- possibly comparing said anti-E1 titers with the anti-E1 titers present during the course of interferon therapy in a sample of a patient which is a long term, sustained responder and of a patient which is a non responder to interferon therapy,
- prognosing the response to interferon treatment of said patient on the basis of the amount anti-E1 titers found in said sample at the start of treatment and/or during the course of treatment.

13. Kit for monitoring or prognosing the response to interferon treatment of patients suffering from HCV infection comprising:

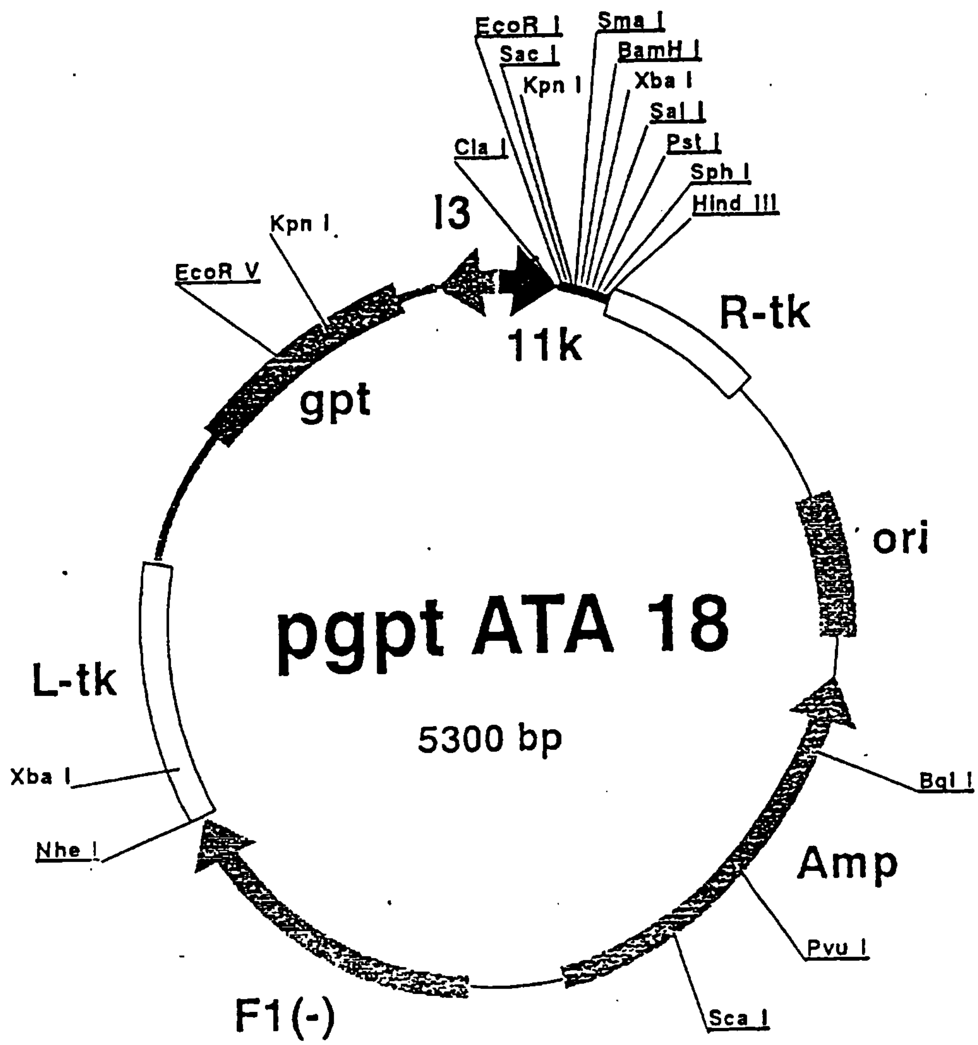
- at least one E1 protein or E1 peptide, more particularly an E1 protein or E1 peptide according to any of claims 1 or 7,
- a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-E1 antibodies present in a biological sample,
- means for detecting the immune complexes formed in the preceding binding reaction,
- possibly also an automated scanning and interpretation device for inferring a decrease of anti-E1 titers during the progression of treatment.

6

ABSTRACT

The present invention relates to a composition comprising essentially purified HCV single envelope proteins selected from the group consisting of E1 and E2, characterized as being isolated in the presence of a means for cleaving disulphide bonds and preferably also in the presence of a means for blocking disulphide bond reformation. The present invention also relates to the diagnostic and therapeutic application of these compositions. Furthermore, the invention relates to the use of HCV E1 protein and peptides for prognosing and monitoring the clinical effectiveness and/or clinical outcome of HCV treatment.

7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EcoR V
11k
R-tk
gpt
ori
pgpt ATA 18
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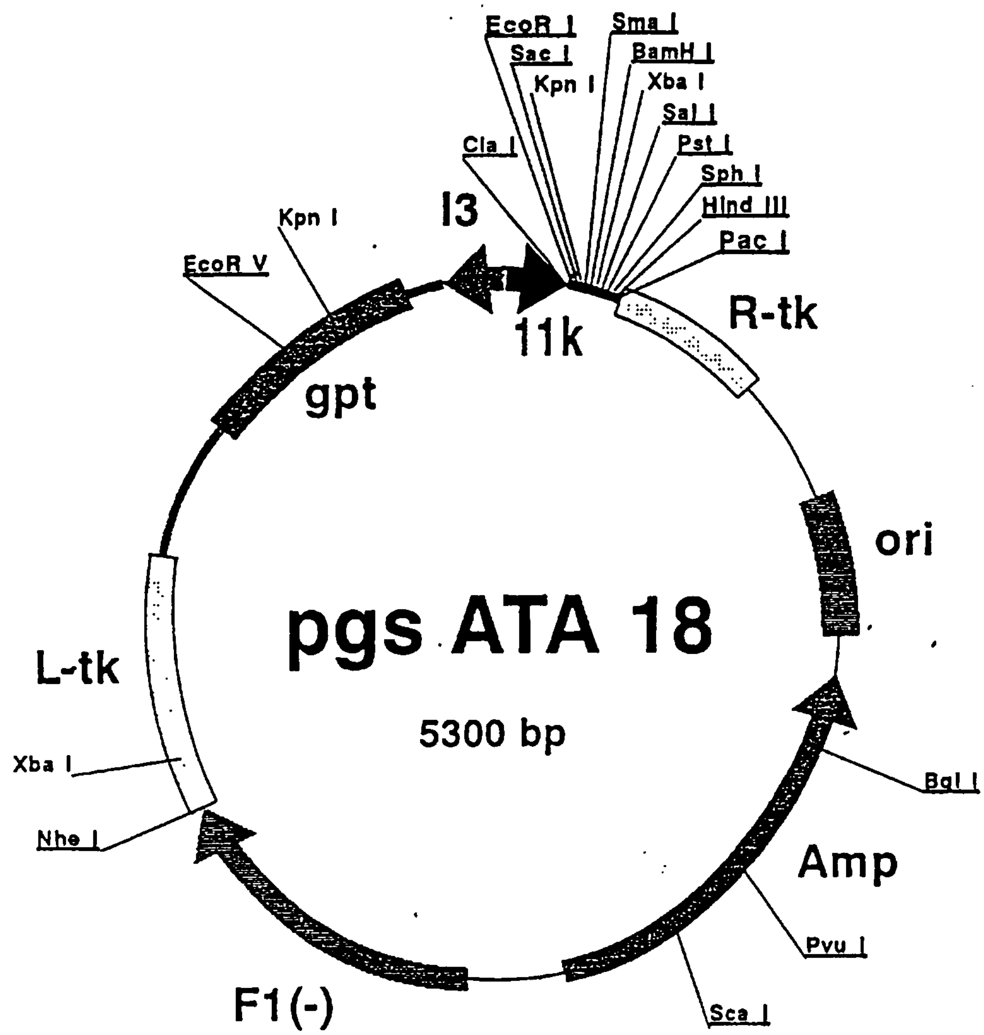
Nhe I
Bgl I
Amp
Pvu I
Sca I
F1(-)

Fig. 1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Kpn I
EcoR V
gpt
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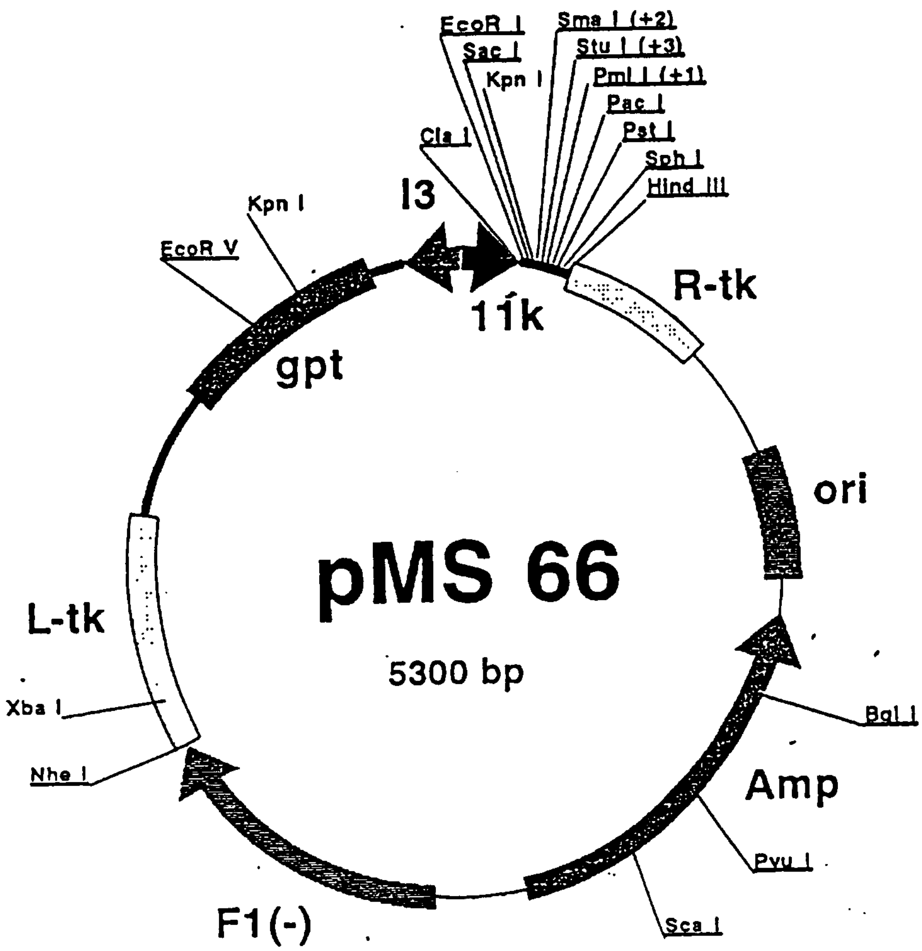
Bgl I
Amp
Pvu I
Sca I
F1(-)

Fig. 2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Cla I
I3
Kpn I
EcoR V
11k
R-tk
gpt
ori
pMS 66
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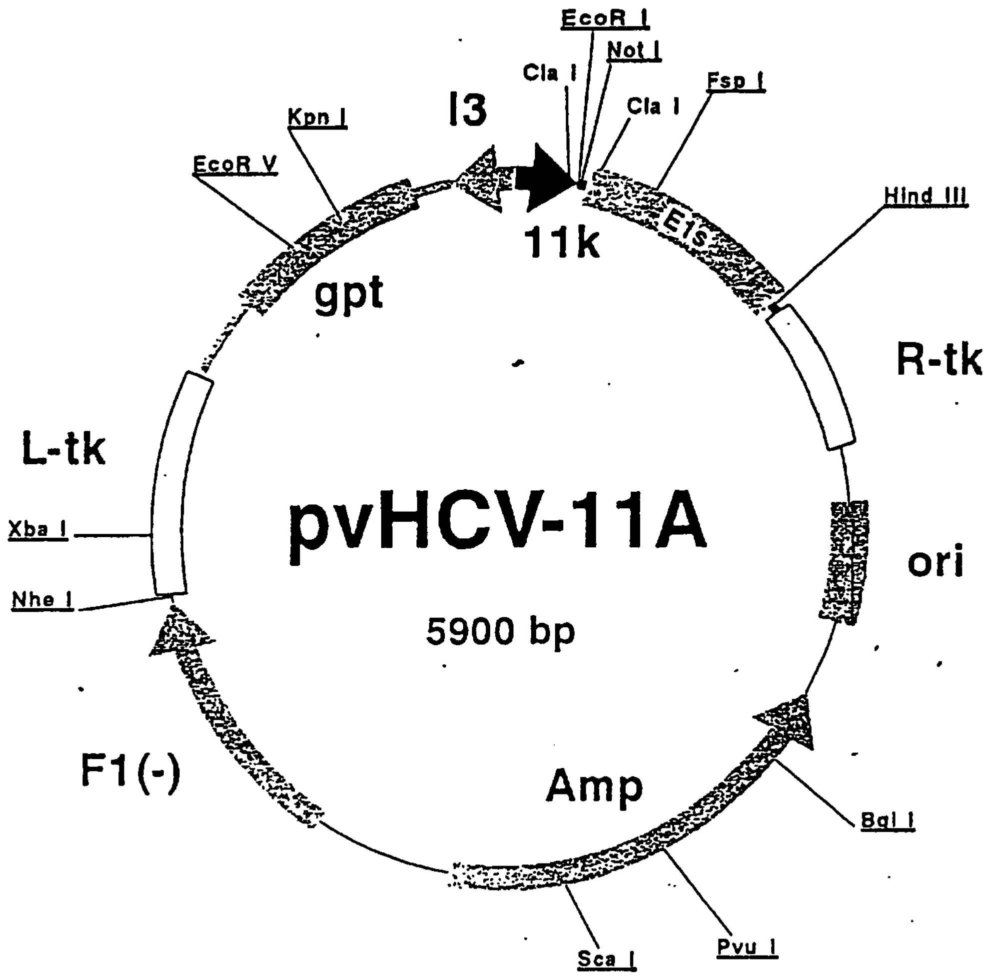
L-tk
Xba I
Nhe I
Bgl I
Amp
Pvu I
Sca I
F1(-)

fig. 3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Not I
Cla I
Cla I
Fsp I
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I3
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11k
E1s
Hind III
gpt
R-tk
L-tk
pvHCV-11A
Xba I
ori
Nhe I
5900 bp
F1(-)
Amp
Bgl I
Sca I
Pvu I

Fig. 4

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Anti-E1 levels in NON-responders to IFN treatment

Series 1

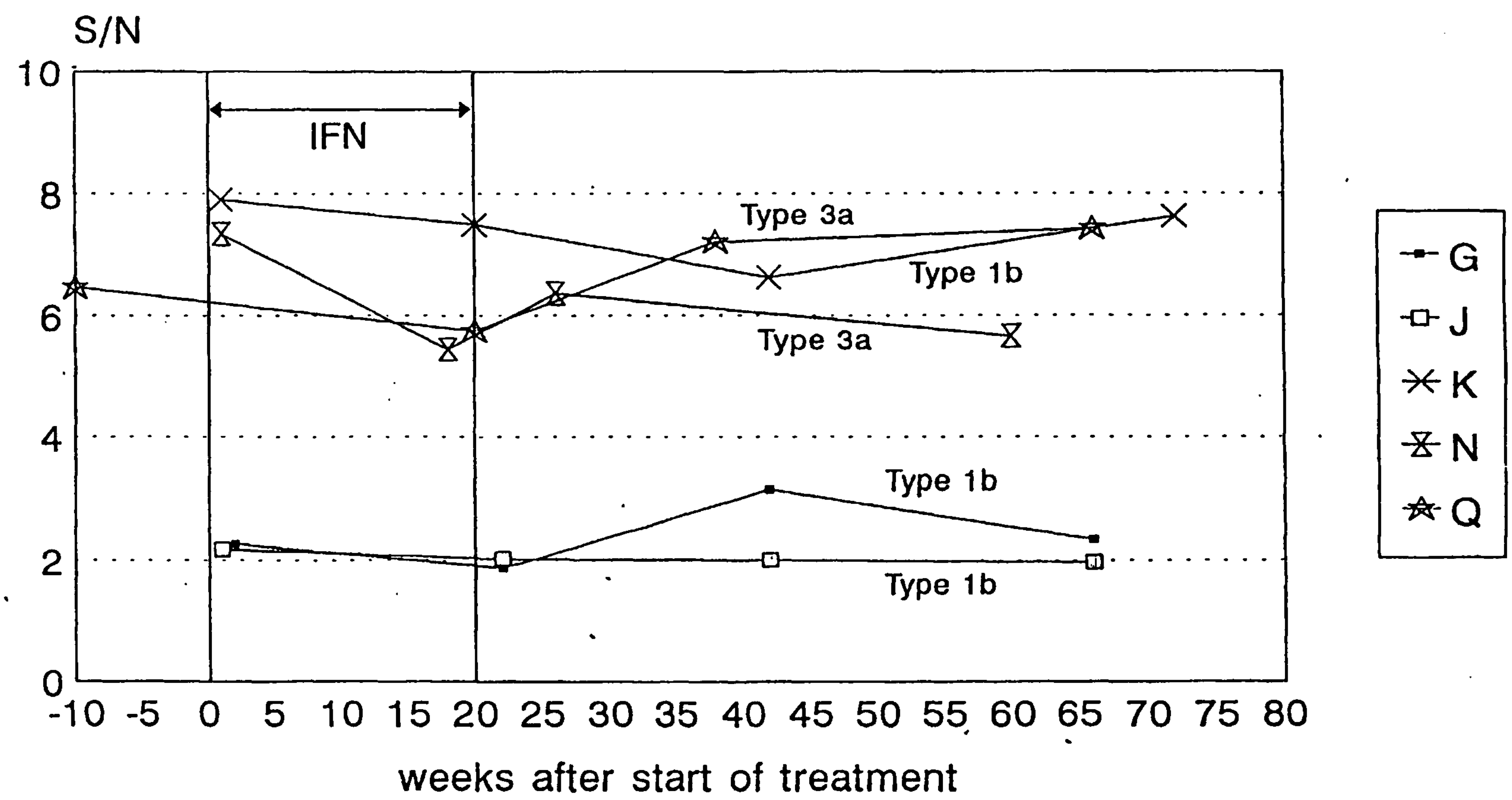

Fig. 5

Anti-E1 levels in RESPONDERS to IFN treatment

Series 1

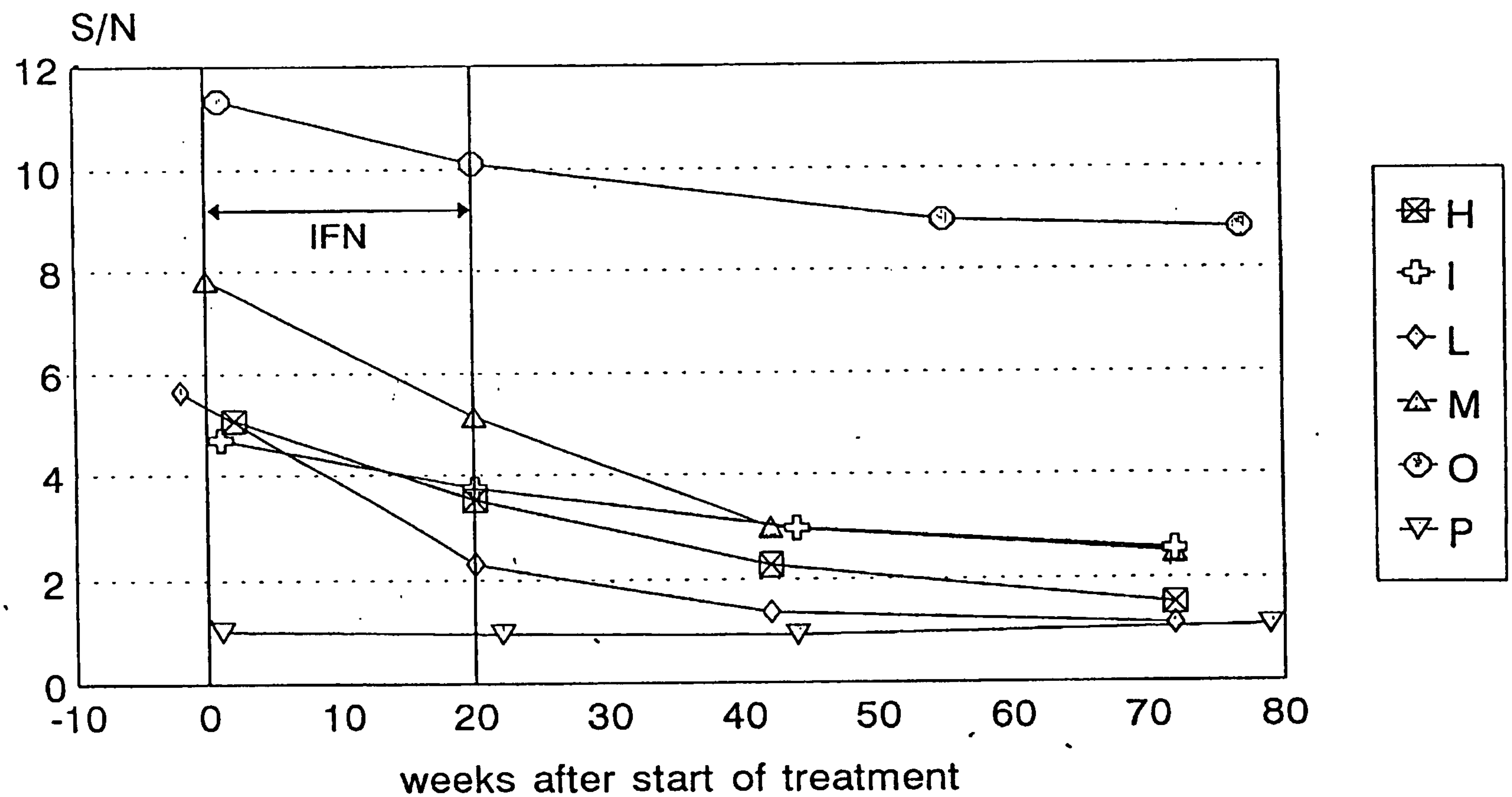

Fig. 6

Anti-E1 levels in patients with COMPLETE response to IFN
Series 2

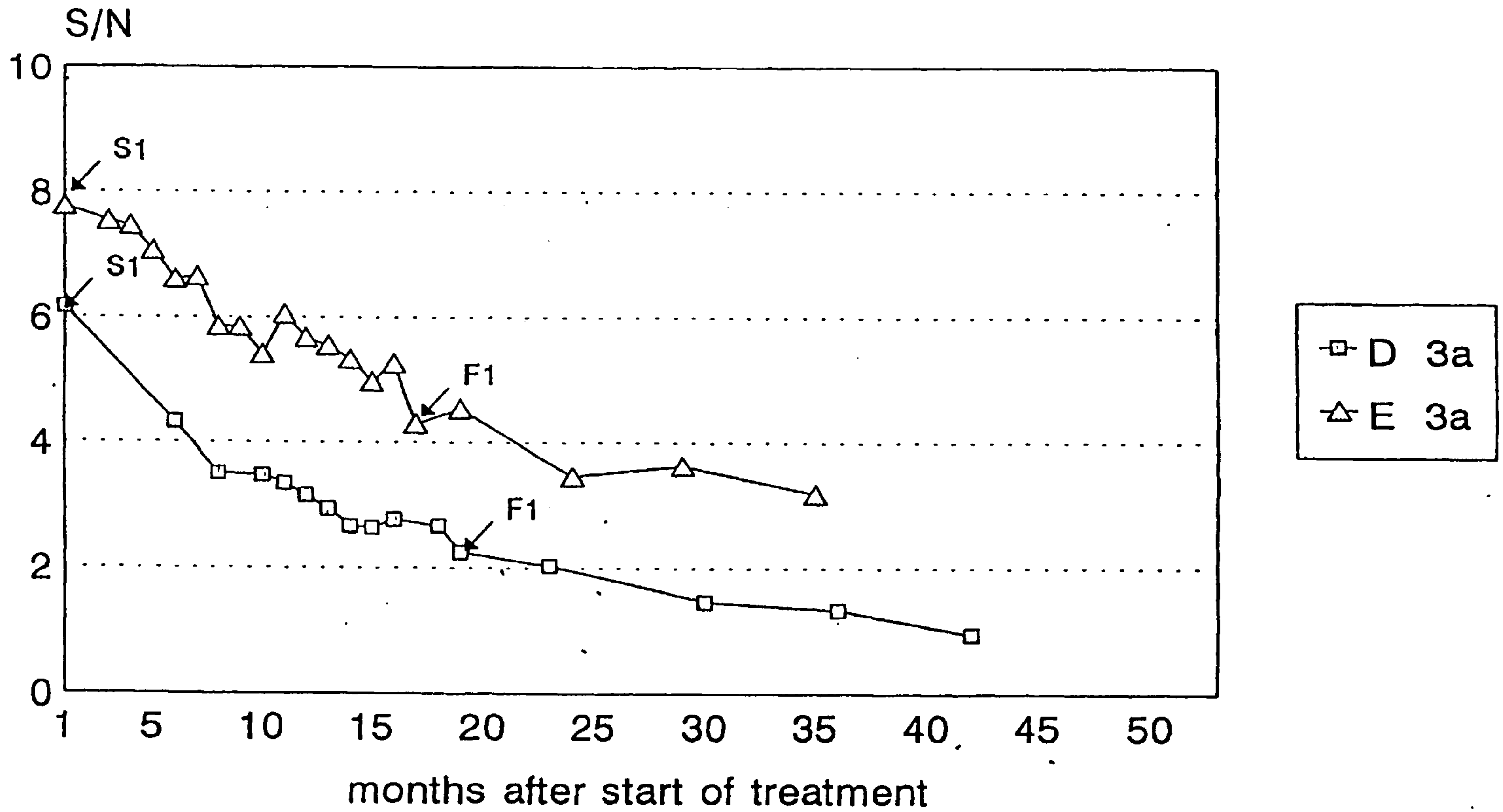

Fig. 7

Anti-E1 levels in INCOMPLETE responders to IFN treatment
Series 2

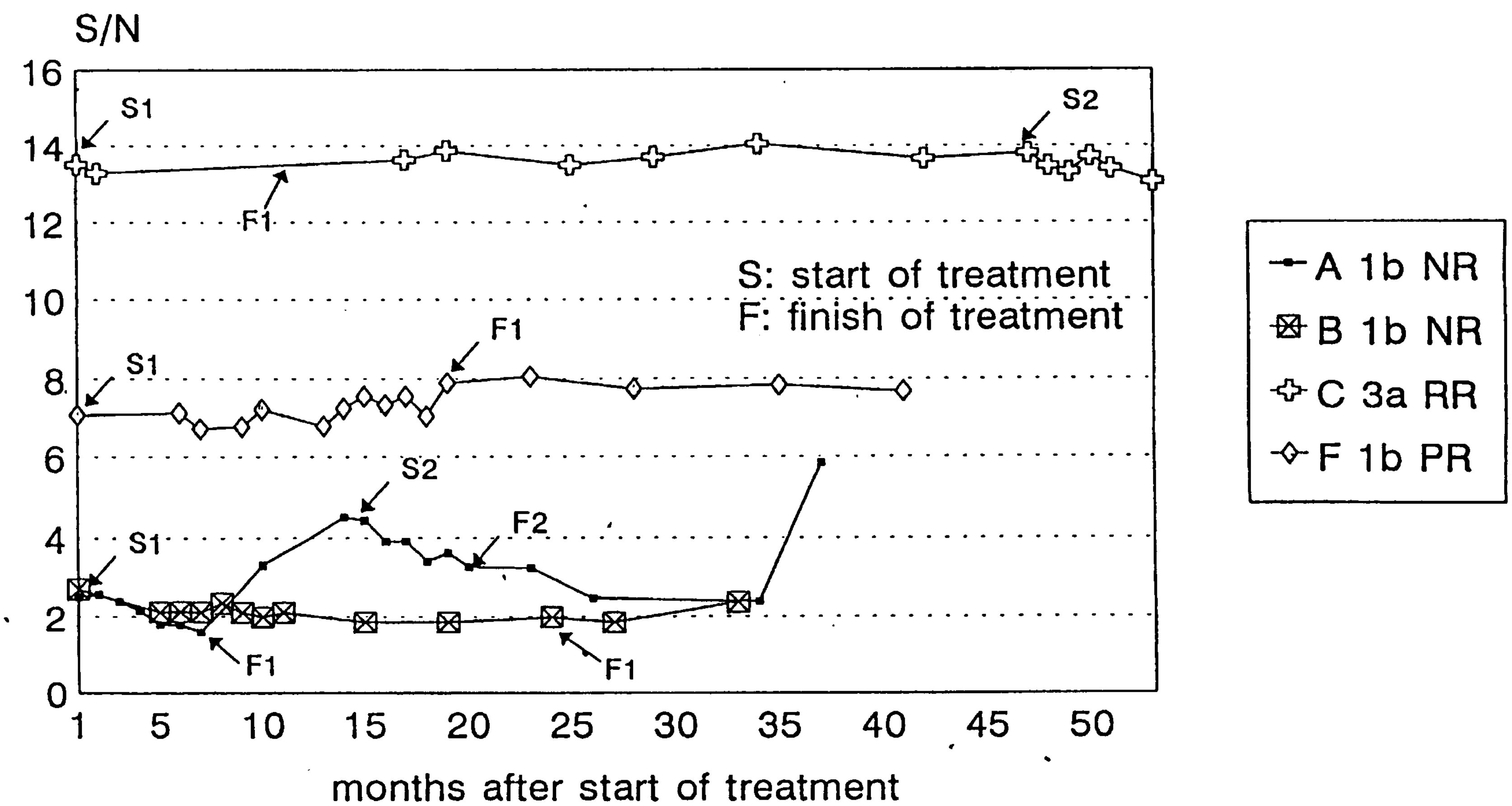

Fig. 8

Anti-E2 levels in NON-RESPONDERS to IFN treatment

Series 1

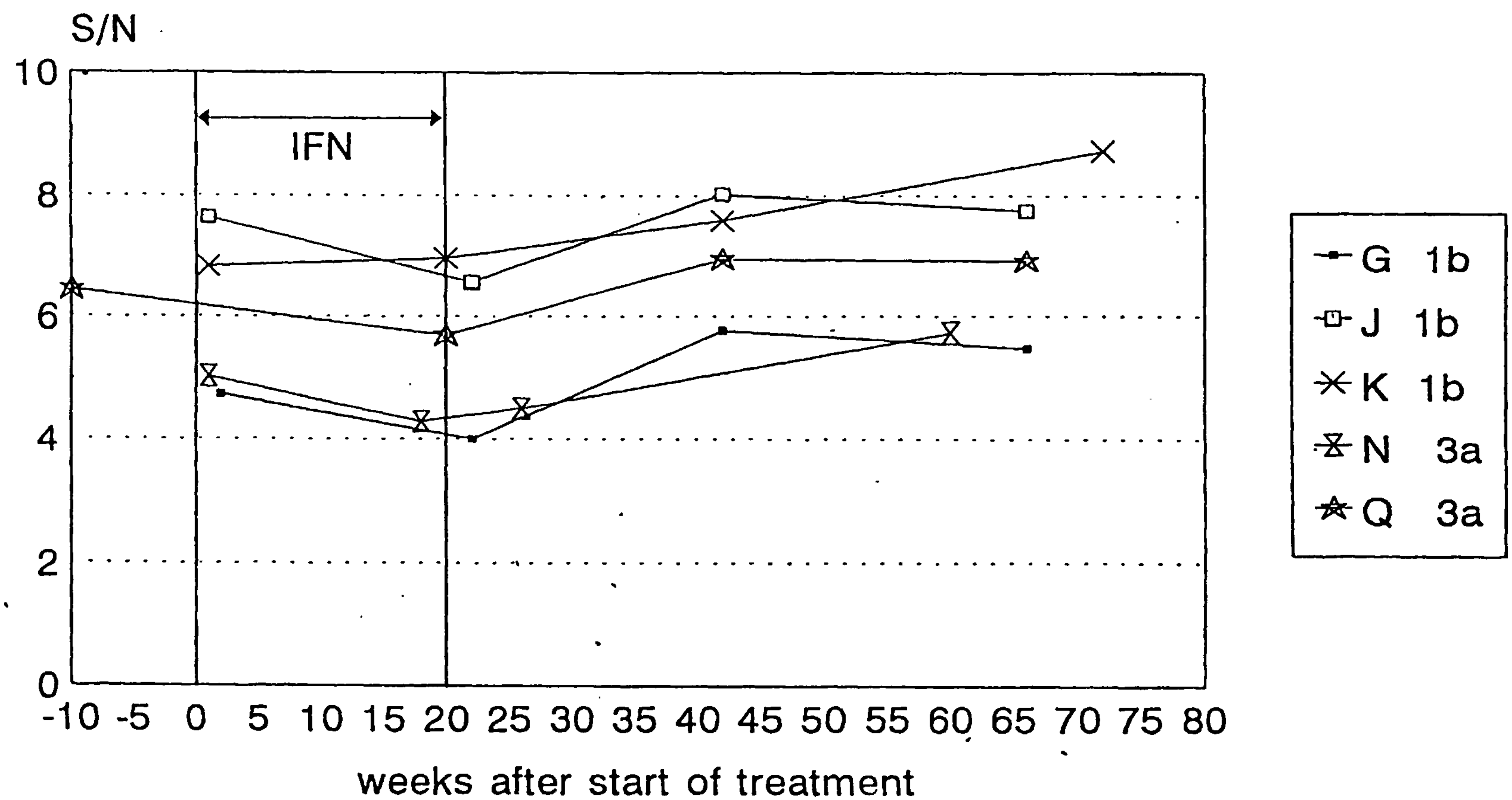

Fig. 9

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Anti-E2 levels in RESPONDERS to IFN treatment
Series 1

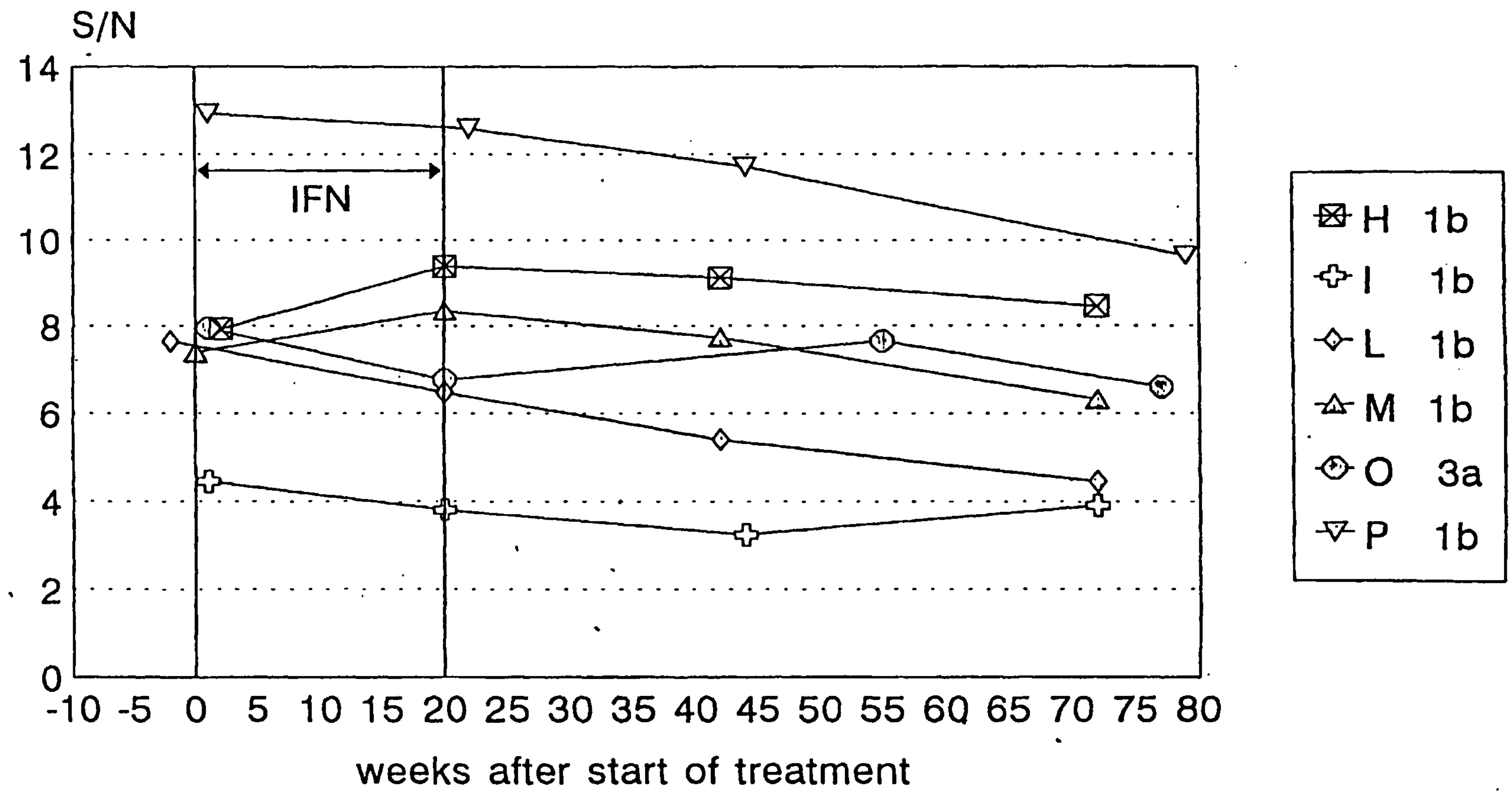


Fig. 10

Anti-E2 levels in INCOMPLETE responders to IFN treatment

Series2

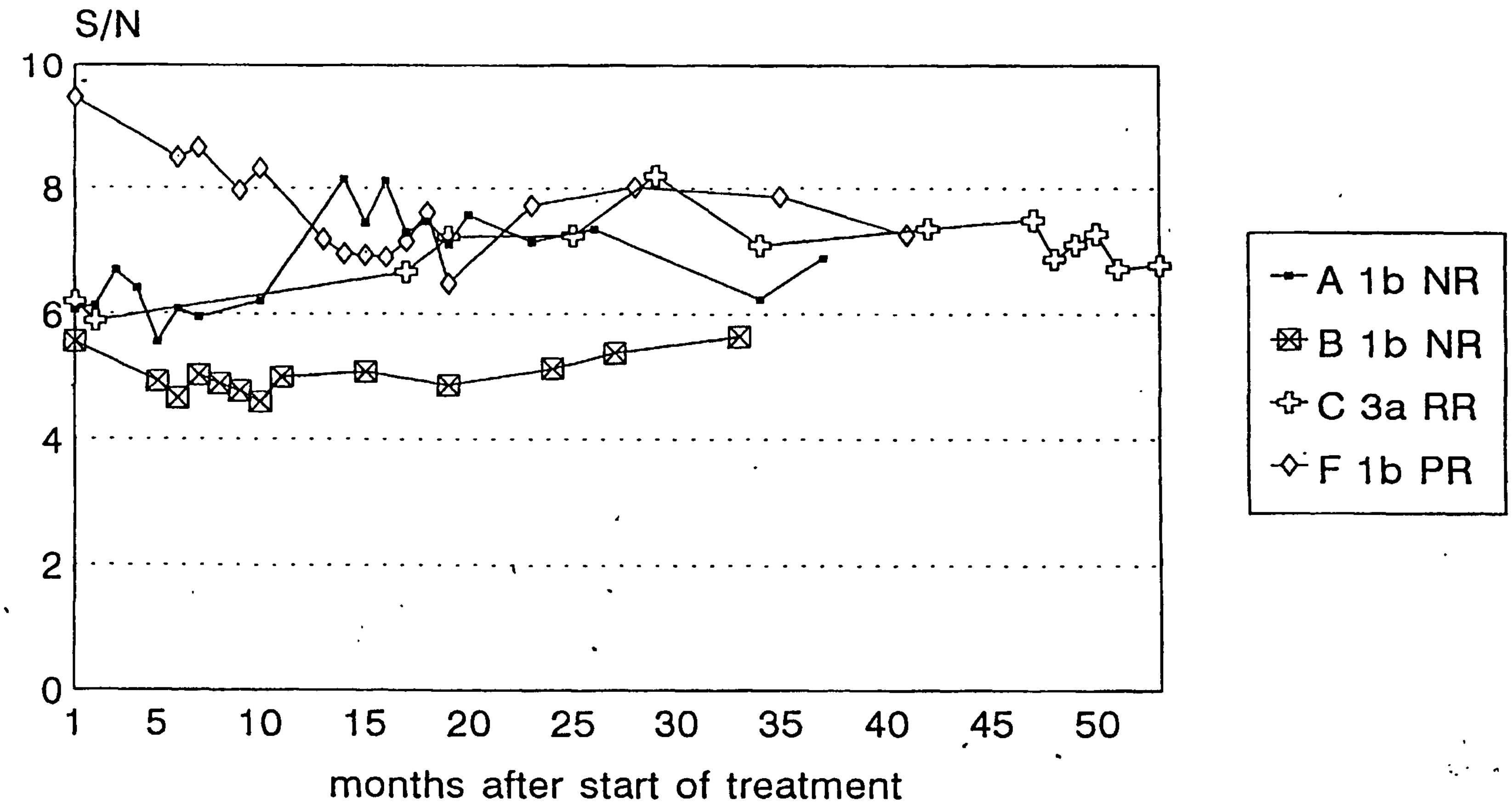


Fig. 11

Anti-E2 levels in COMPLETE responders to IFN treatment

Series 2

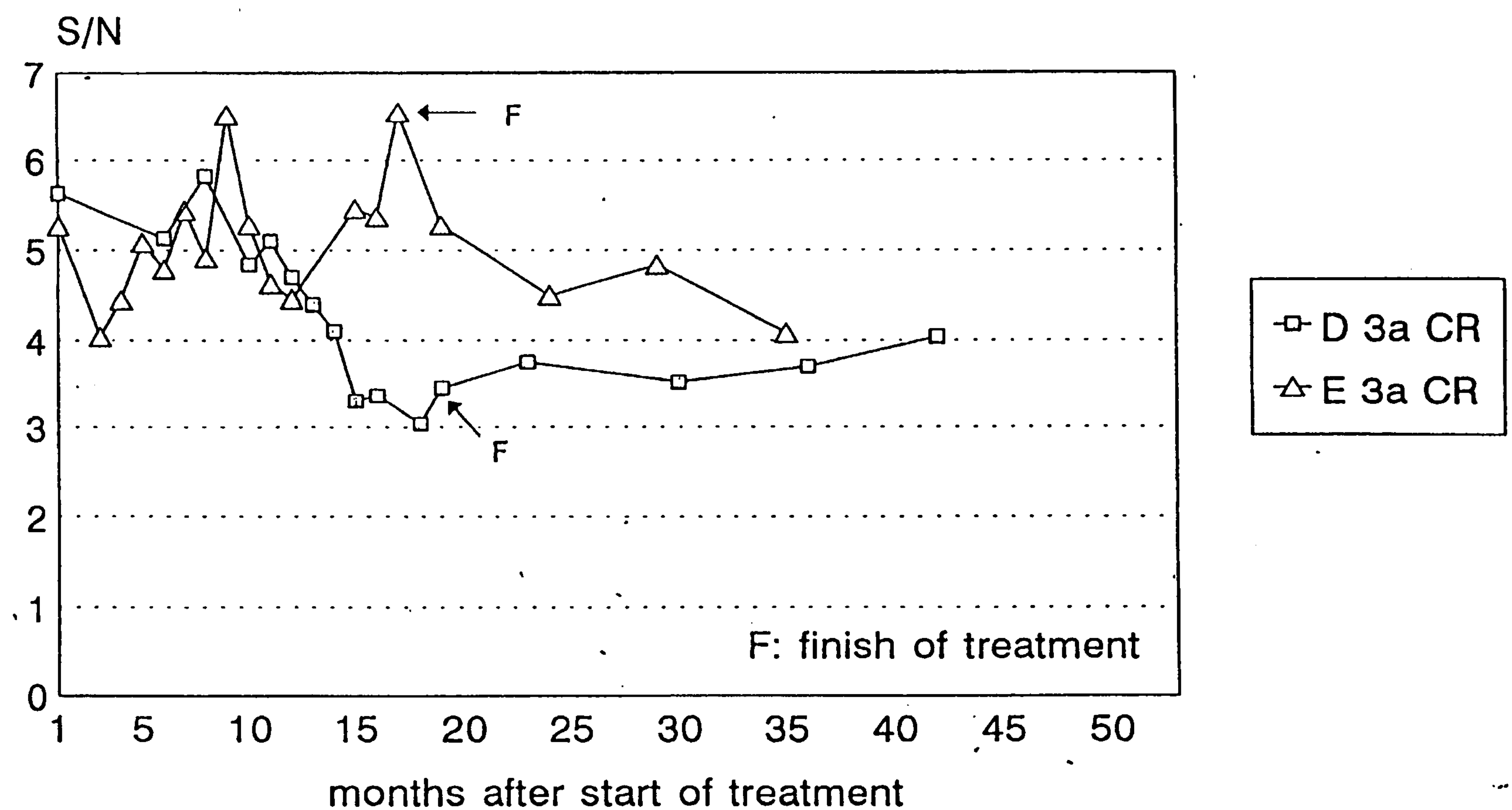


Fig. 12

FIGURE 13

Human anti-E1 reactivity competed with peptides

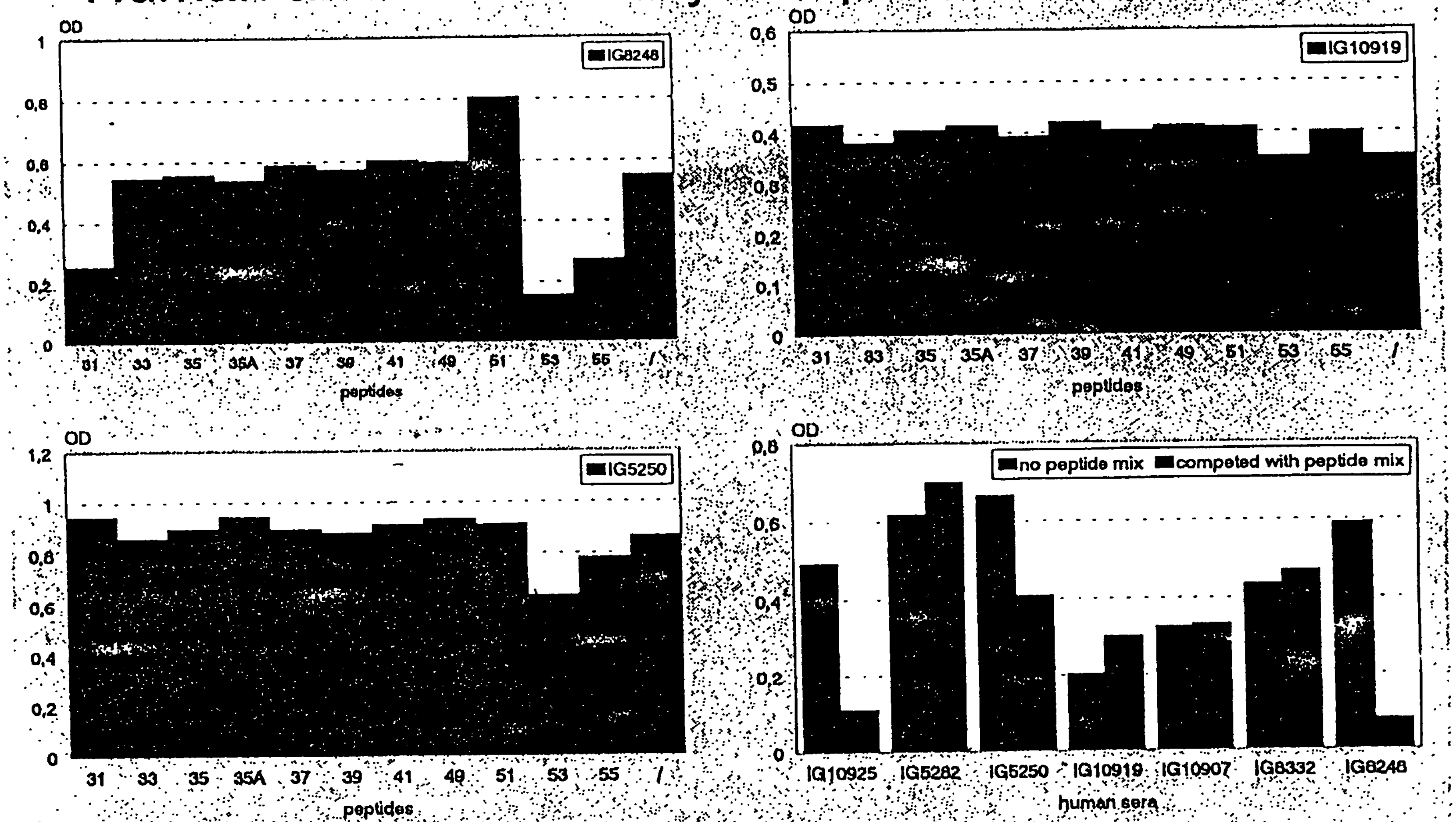
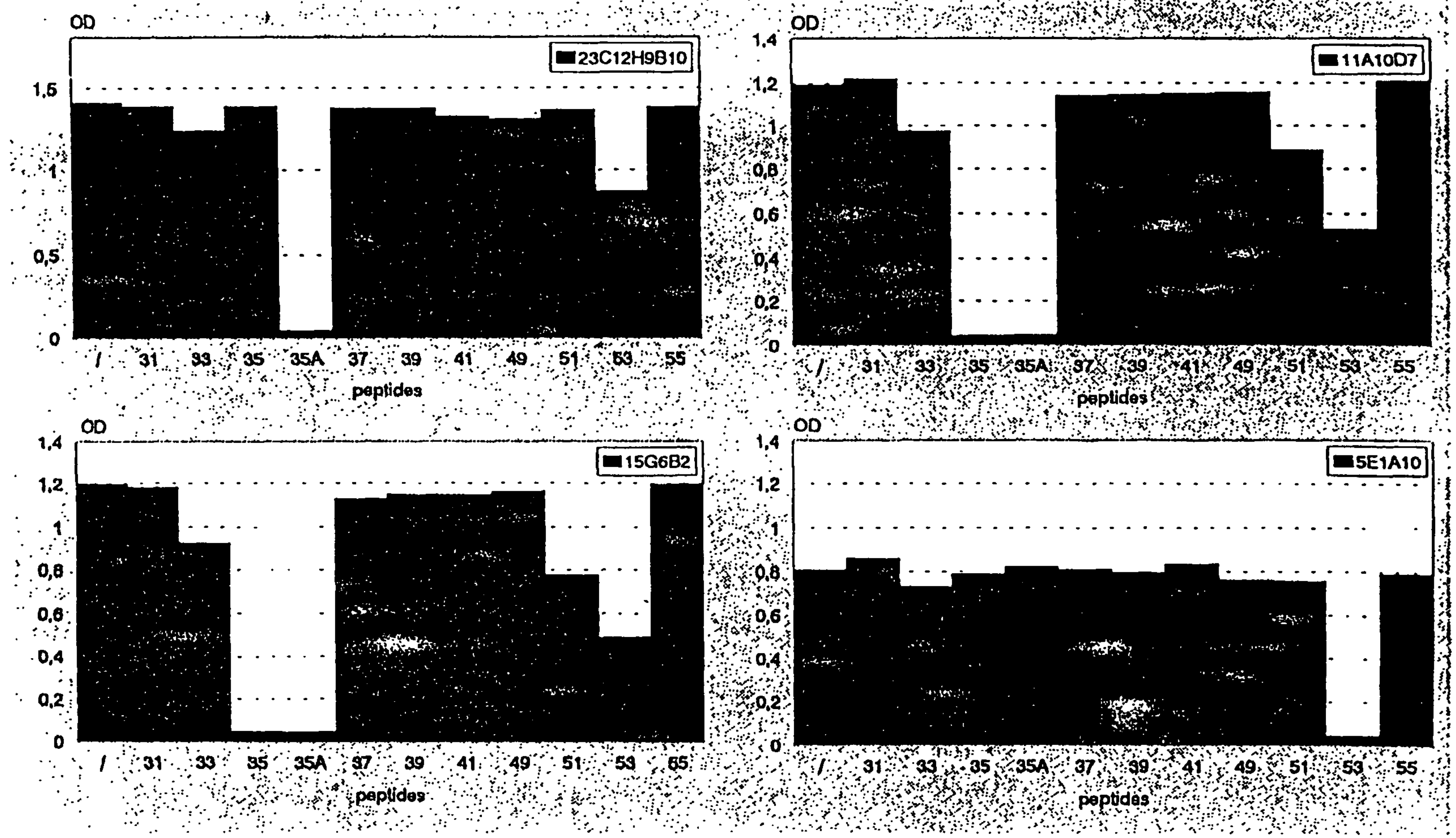


FIGURE 14

Competition of reactivity of anti-E1 Mabs with peptides



Anti-E1 (epitope 1) levels in NON-RESPONDERS to IFN treatment

Series 1

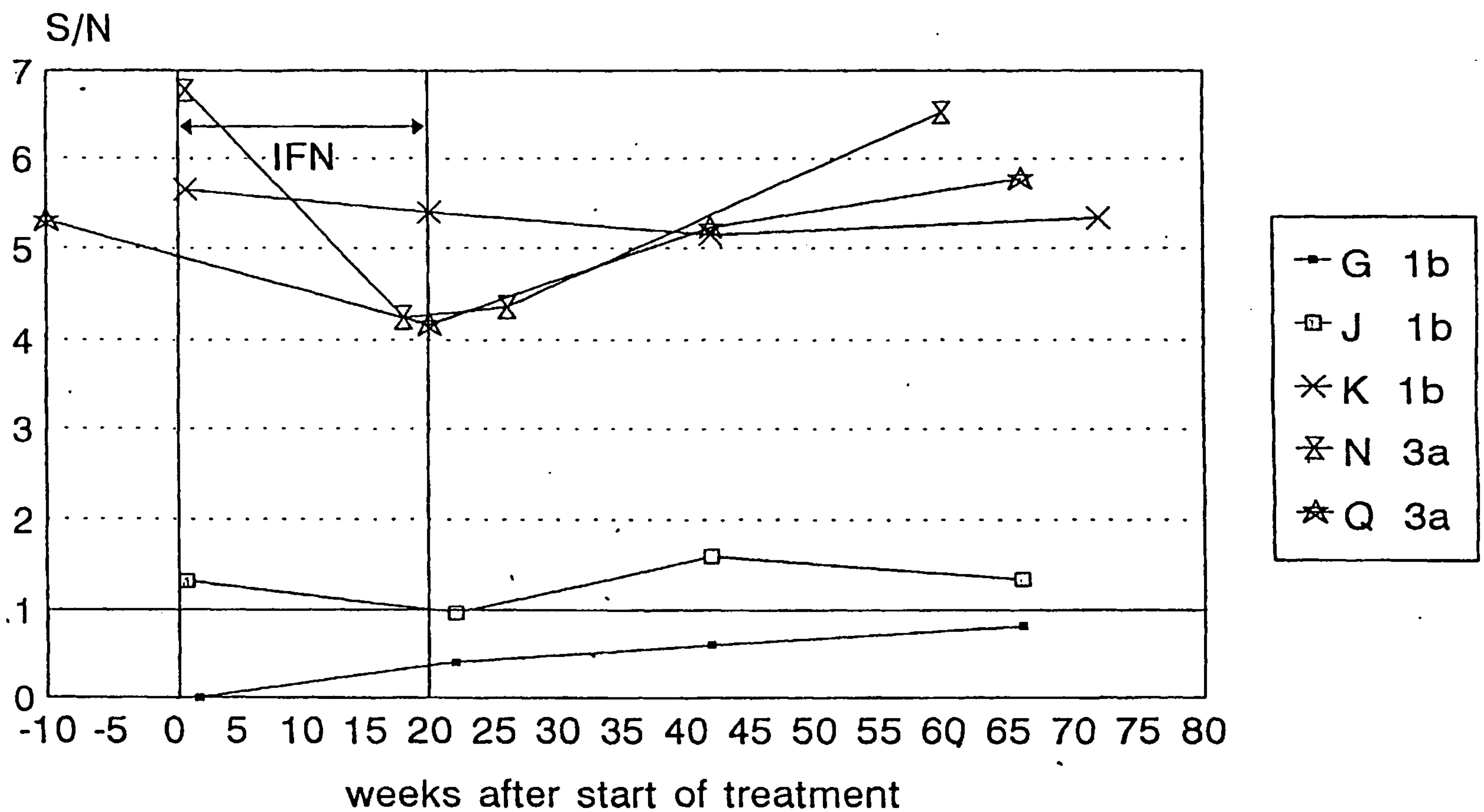


Fig. 15

Anti-E1 (epitope 1) levels in RESPONDERS to IFN treatment

Series 1

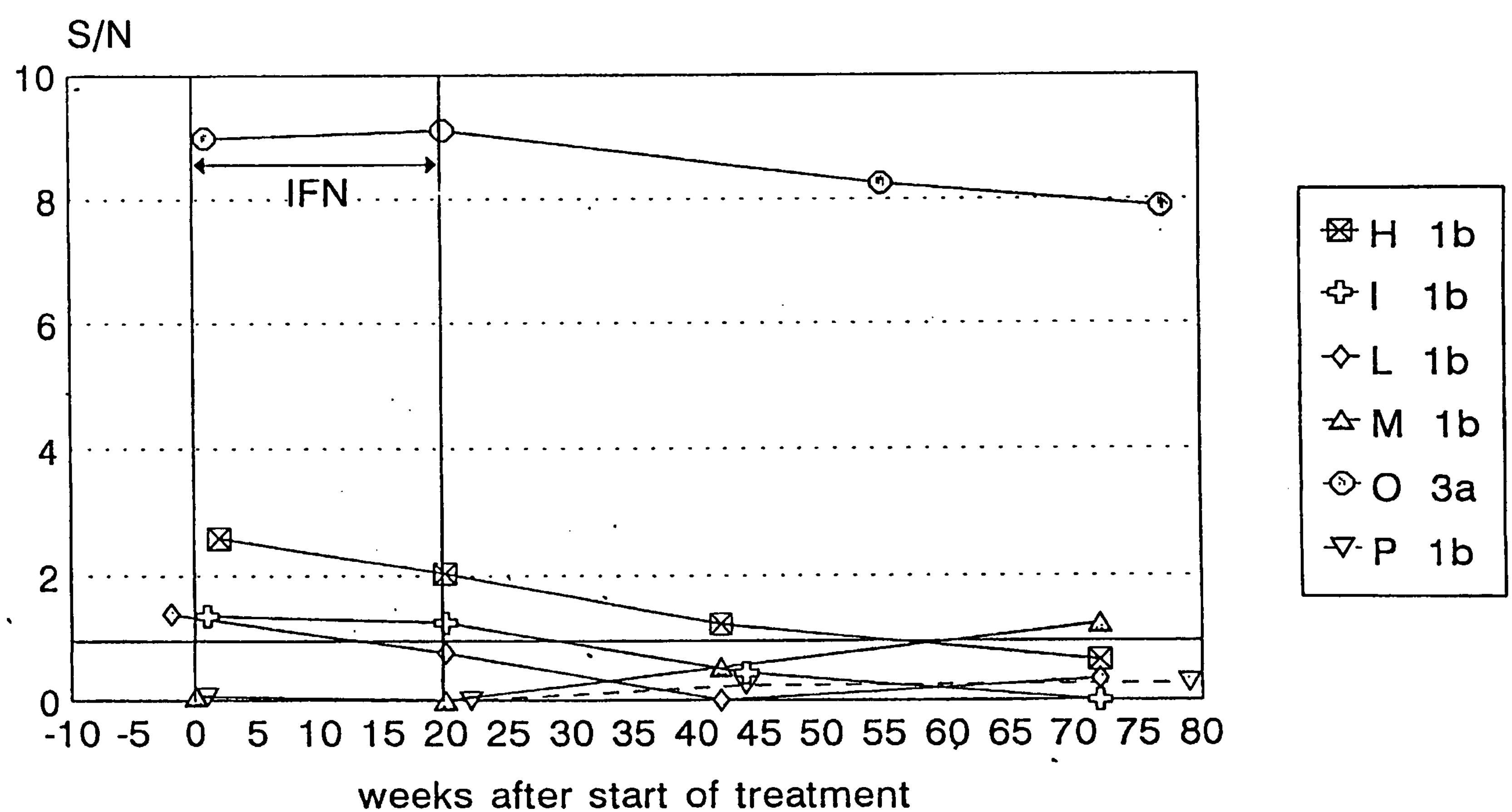


Fig. 16

Anti-E1 (epitope 2) levels in NON-RESPONDERS to IFN treatment
Series 1

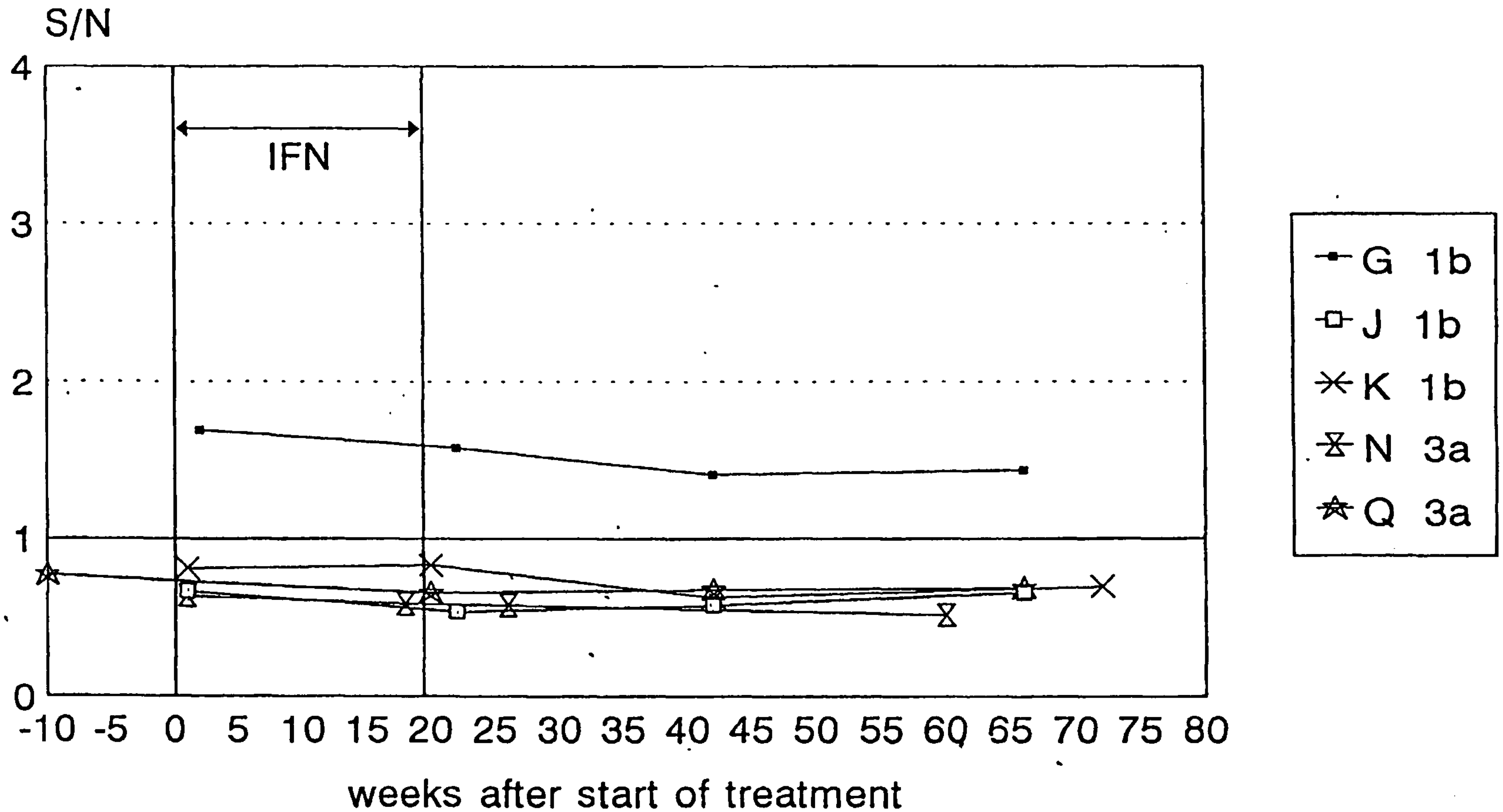


Fig. 17

Anti-E1 (epitope 2) levels in RESPONDERS to IFN treatment
Series 1

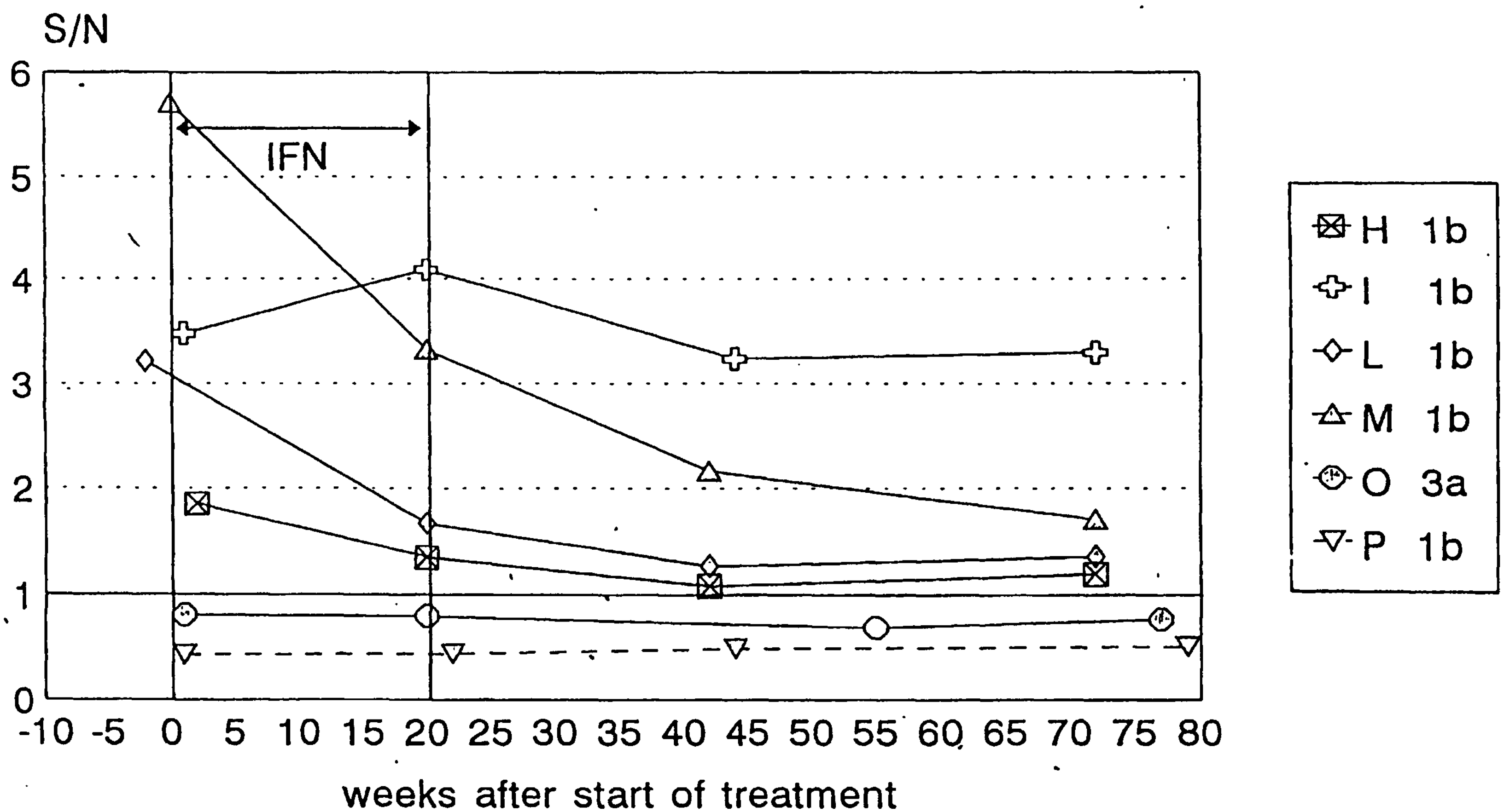


Fig. 18

FIGURE 19

Competition of reactivity of anti-E2 Mabs with peptides

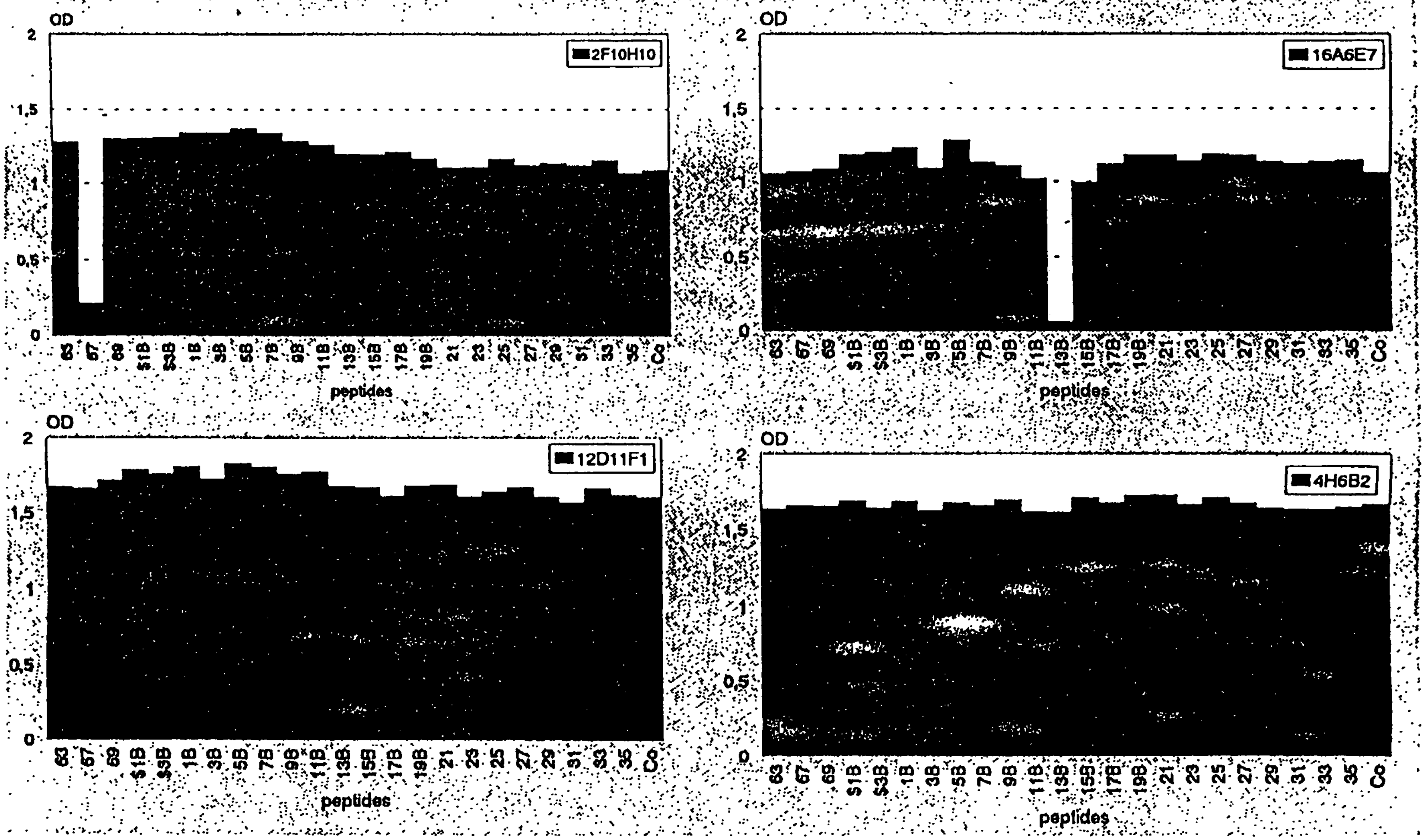


FIGURE 20

Human anti-E2 reactivity competed with peptides

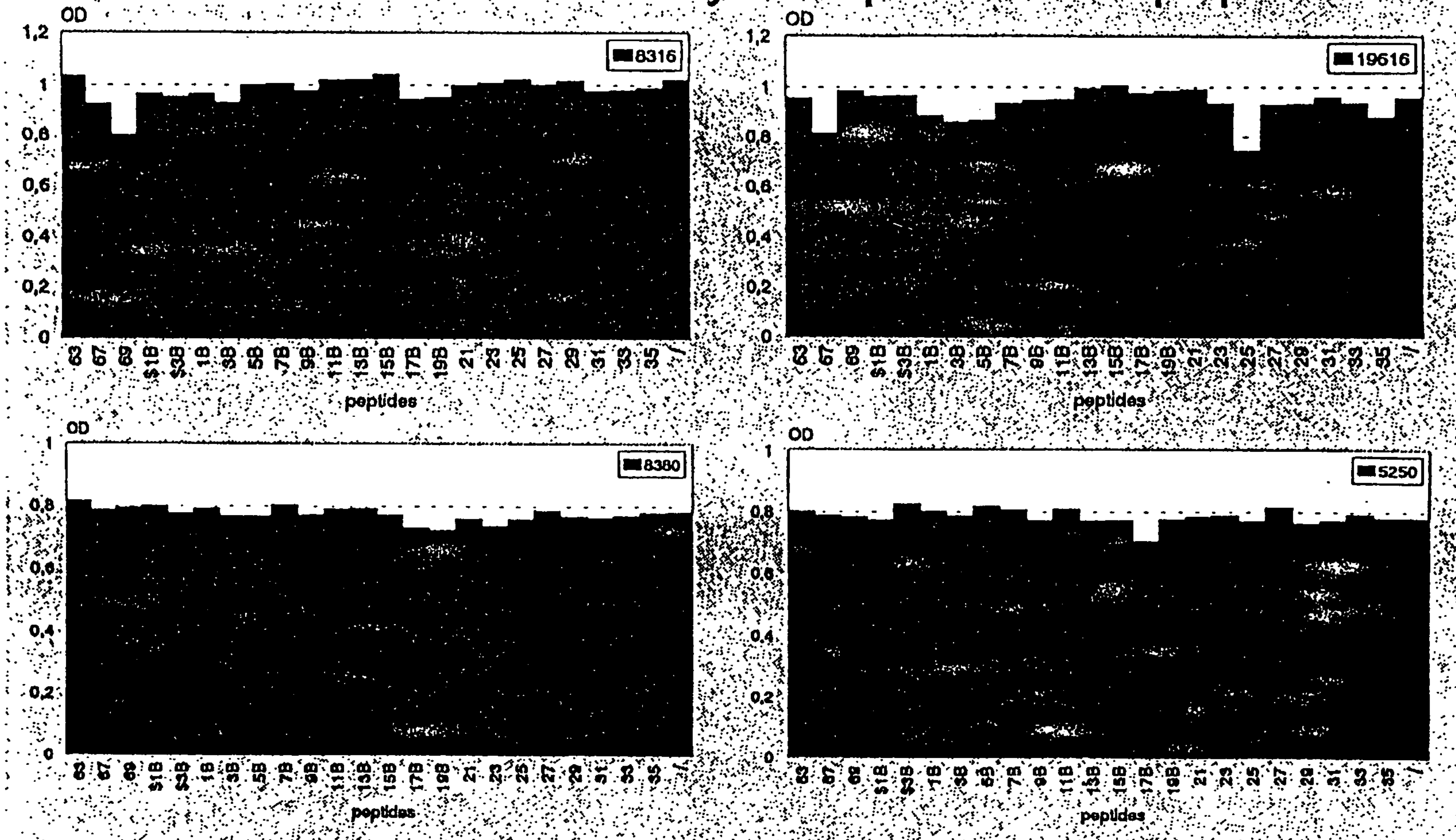


Figure 21

SEQ ID NO. 1
5' GGCATGCAAGCTTAATTAATT 3'
3' ACGTCCGTACGTTCGAATTAATTAATCGA 5'

SEQ ID NO. 2
5'CCGGGGAGGCCTGCACGTGATCGAGGGCAGACACCATCACCACCATCACTAATAGT
TAATTAACTGCA 3'
3
CCTCCGGACGTGCACTAGCTCCCGTCTGTGGTAGTGGTGGTAGTGATTATCAATTAATTG
5'

SEQ ID NO. 3 (HCCI9A)
ATGCCCGGTTGCTCTTTCTCTATCTTCCTCTTGGCTTTACTGTCCTGTCTGACCATTCCA
GCTTCCGCTTATGAGGTGCGCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCT
CCAACTCAAGCATTGTGTATGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCG
TGCCCTGCGTTCGGGAGAACAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCT
CGCAGCTAGGAACGCCAGCGTCCCCACCACGACAATACGACGCCACGTCGATTTGCT
CGTTGGGGCGGCTGCTCTCTGTTCCGCTATGTACGTGGGGATCTCTGCGGATCTGTCT
TCCTCGTCTCCCAGCTGTTCACCATCTCGCCTCGCCGGCATGAGACGGTGCAGGACTG
CAATTGCTCAATCTATCCCGGCCACATAACAGGTCACCGTATGGCTTGGGATATGATG
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GCTGTCGTGGACATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGGGCCTCGCCTAC
TATTCCATGGTGGGGAACTGGGCTAAGGTTTTGATTGTGATGCTACTCTTTGCTCTCTA
ATAG

SEQ ID NO. 4 (HCCI10A)
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TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
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CGGGGGCCCATTGGGGAGTCCTGGCGGGTCTCGCCTACTATTCCATGGTGGGGAACTG
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SEQ ID NO. 5 (HCCI11A)
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CAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTAT
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GTCCCCACTACGACAATACGACGCCACGTCGATTTGCTCGTTGGGGCGGCTGCTTTCT

GTTCCGCTATGTACGTGGGGGATCTCTGCGGATCTGTCTTCCTCGTCTCCCAGCTGTTC
ACCATCTCGCCTCGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCCG
GCCACATAACAGGTCACCGTATGGCTTGGGATATGATGATGAACTGGTAATAG

SEQ ID NO. 6 (HCCI12A)
ATGCCCGGTTGCTCTTTCTCTATCTTCCTCTTGGCCCTGCTGTCCTGTCTGACCATACC
AGCTTCCGCTTATGAAGTGCGCAACGTGTCCGGGGTGTACCATGTCACGAACGACTGC
TCCAACTCAAGCATAGTGTATGAGGCAGCGGACATGATCATGCACACCCCCGGGTGC
GTGCCCTGCGTTCGGGAGGGCAACTCCTCCCGTTGCTGGGTGGCGCTCACTCCCACGC
TCGCGGCCAGGAACGCCAGCGTCCCCACAACGACAATACGACGCCACGTCGATTTGC
TCGTTGGGGCTGCTGCTTTCTGTTCCGCTATGTACGTGGGGGATCTCTGCGGATCTGTT
TTCCTTGTTTCCCAGCTGTTCACCTTCTCACCTCGCCGGCATCAAACAGTACAGGACTG
CAACTGCTCAATCTATCCCGGCCATGTATCAGGTCACCGCATGGCTTGGGATATGATG
ATGAACTGGTCCTAATAG

SEQ ID NO. 7 (HCCI13A)
ATGTCCGGTTGCTCTTTCTCTATCTTCCTCTTGGCCCTGCTGTCCTGTCTGACCATACC
AGCTTCCGCTTATGAAGTGCGCAACGTGTCCGGGGTGTACCATGTCACGAACGACTGC
TCCAACTCAAGCATAGTGTATGAGGCAGCGGACATGATCATGCACACCCCCGGGTGC
GTGCCCTGCGTTCGGGAGGGCAACTCCTCCCGTTGCTGGGTGGCGCTCACTCCCACGC
TCGCGGCCAGGAACGCCAGCGTCCCCACAACGACAATACGACGCCACGTCGATTTGC
TCGTTGGGGCTGCTGCTTTCTGTTCCGCTATGTACGTGGGGGATCTCTGCGGATCTGTT
TTCCTTGTTTCCCAGCTGTTCACCTTCTCACCTCGCCGGCATCAAACAGTACAGGACTG
CAACTGCTCAATCTATCCCGGCCATGTATCAGGTCACCGCATGGCTTGGGATATGATG
ATGAACTGGTAATAG

SEQ ID NO. 8 (HCCI17A)
ATGCTGGGTAAGGCCATCGATACCCTTACGTGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAAGACGGCGTGAACTATGCAACAGGGAATTTGCCTGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTACTGTCCTGTCTAACCATTCCAGCTTCCGCTTACGAGGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCGGCTAGGAACGCCAG
CATCCCCACTACAACAATACGACGCCACGTCGATTTGCTCGTTGGGGCGGCTGCTTTC
TGTTCCGCTATGTACGTGGGGGATCTCTGCGGATCTGTCTTCCTCGTCTCCCAGCTGTT
CACCATCTCGCCTCGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCC
GGCCACATAACGGGTCACCGTATGGCTTGGGATATGATGATGAACTGGTACTAATAG

SEQ ID NO. 9 (HCPr51)
ATGCCCGGTTGCTCTTTCTCTATCTT

SEQ ID NO. 10 (HCPr52)
ATGTTGGGTAAGGTCATCGATACCCT

SEQ ID NO. 11 (HCPr53)
CTATTAGGACCAGTTCATCATCATATCCCA

SEQ ID NO. 12 (HCPr54)
CTATTACCAGTTCATCATCATATCCCA

SEQ ID NO. 13 (HCPr107)
ATACGACGCCACGTCGATTCCCAGCTGTTCACCATC

SEQ ID NO. 14 (HCPr108)
GATGGTGAACAGCTGGGAATCGACGTGGCGTCGTAT

SEQ ID NO. 15 (HCCI37)
ATGTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGAACGCCAG
CGTCCCCACCACGACAATACGACGCCACGTCGATTCCCAGCTGTTCACCATCTCGCCT
CGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCCGGCCACATAACG
GGTCACCGTATGGCTTGGGATATGATGATGAACTGGTCGCCTACAACGGCCCTGGTGG
TATCGCAGCTGCTCCGGATCCCACAAGCTGTCGTGGACATGGTGGCGGGGGCCCATTG
GGGAGTCCTGGCGGGTCTCGCCTACTATTCCATGGTGGGGAACTGGGCTAAGGTTTTG
ATTGTGATGCTACTCTTTGCTCCCTAATAG

SEQ ID NO. 16 (HCCI38)
ATGTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGAACGCCAG
CGTCCCCACCACGACAATACGACGCCACGTCGATTCCCAGCTGTTCACCATCTCGCCT
CGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCCGGCCACATAACG
GGTCACCGTATGGCTTGGGATATGATGATGAACTGGTAA
TAG

SEQ ID NO. 17 (HCCI39)
ATGTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGAACGCCAG
CGTCCCCACCACGACAATACGACGCCACGTCGATTCCCAGCTGTTCACCATCTCGCCT
CGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCCGGCCACATAACG
GGTCACCGTATGGCTTGGGATATGATGATGAACTGGTCGCCTACAACGGCCCTGGTGG
TATCGCAGCTGCTCCGGATCCTCTAATAG

SEQ ID NO. 18 (HCCI40)
ATGTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGAACGCCAG
CGTCCCCACCACGACAATACGACGCCACGTCGATTCCCAGCTGTTCACCATCTCGCCT

CGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCCGGCCACATAACG
GGTCACCGTATGGCTTGGGATATGATGATGAACTGGTCGCCTACAACGGCCCTGGTGG
TATCGCAGCTGCTCCGGATCGTGATCGAGGGCAGACACCATCACCACCATCACTAATAG

SEQ ID NO. 19 (HCCI62)
ATGGGTAAGGTCATCGATACCCTTACGTGCGGATTCGCCGATCTCATGGGGTACATCC
CGCTCGTCGGCGCTCCCGTAGGAGGCGTCGCAAGAGCCCTTGCGCATGGCGTGAGGG
CCCTTGAAGACGGGATAAATTTCGCAACAGGGAATTTGCCCGGTTGCTCCTTTTCTAT
TTTCCTTCTCGCTCTGTTCTCTTGCTTAATTCATCCAGCAGCTAGTCTAGAGTGGCGGA
ATACGTCTGGCCTCTATGTCCTTACCAACGACTGTTCCAATAGCAGTATTGTGTACGA
GGCCGATGACGTTATTCTGCACACACCCGGCTGCATACCTTGTGTCCAGGACGGCAAT
ACATCCACGTGCTGGACCCCAGTGACACCTACAGTGGCAGTCAAGTACGTCGGAGCA
ACCACCGCTTCGATACGCAGTCATGTGGACCTATTAGTGGGCGCGGCCACGATGTGCT
CTGCGCTCTACGTGGGTGACATGTGTGGGGCTGTCTTCCTCGTGGGACAAGCCTTCAC
GTTCAGACCTCGTCGCCATCAAACGGTCCAGACCTGTAACTGCTCGCTGTACCCAGGC
CATCTTTCAGGACATCGAATGGCTTGGGATATGATGATGAACTGGTAATAG

SEQ ID NO. 20 (HCCI63)
ATGGGTAAGGTCATCGATACCCTAACGTGCGGATTCGCCGATCTCATGGGGTATATGC
CGCTCGTAGGCGGCCCCATTGGGGGCGTCGCAAGGGCTCTCGCACACGGTGTGAGGG
TCCTTGAGGACGGGGTAAACTATGCAACAGGGAATTTACCCGGTTGCTCTTTCTCTAT
CTTTATTCTTGCTCTTCTCTCGTGTCTGACCGTTCCGGCCTCTGCAGTTCCCTACCGAA
ATGCCTCTGGGATTTATCATGTTACCAATGATTGCCCAAACTCTTCCATAGTCTATGAG
GCAGATAACCTGATCCTACACGCACCTGGTTGCGTGCCTTGTGTCATGACAGGTAATG
TGAGTAGATGCTGGGTCCAAATTACCCCTACACTGTCAGCCCCGAGCCTCGGAGCAGT
CACGGCTCCTCTTCGGAGAGCCGTTGACTACCTAGCGGGAGGGGCTGCCCTCTGCTCC
GCGTTATACGTAGGAGACGCGTGTGGGGCACTATTCTTGGTAGGCCAAATGTTCACCT
ATAGGCCTCGCCAGCACGCTACGGTGCAGAACTGCAACTGTTCCATTTACAGTGGCCA
TGTTACCGGCCACCGGATGGCATGGGATATGATGATGAACTGGTAATAG

SEQ ID NO. 21 (HCPr109)
TGGGATATGATGATGAACTGGTC

SEQ ID NO. 22 (HCPr72)
CTATTATGGTGGTAAKGCCARCARGAGCAGGAG

SEQ ID NO. 23 (HCCL22A)
TGGGATATGATGATGAACTGGTCGCCTACAACGGCCCTGGTGGTATCGCAGCTGCTCC
GGATCCCACAAGCTGTCGTGGACATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGG
GCCTCGCCTACTATTCCATGGTGGGGAACTGGGCTAAGGTTTTGGTTGTGATGCTACT
CTTTGCCGGCGTCGACGGGCATACCCGCGTGTCAGGAGGGGCAGCAGCCTCCGATAC
CAGGGGCCTTGTGTCCCTCTTTAGCCCCGGGTCGGCTCAGAAAATCCAGCTCGTAAAC
ACCAACGGCAGTTGGCACATCAACAGGACTGCCCTGAACTGCAACGACTCCCTCCAA
ACAGGGTTCTTTGCCGCACTATTCTACAAACACAAATTCAACTCGTCTGGATGCCCAG
AGCGCTTGGCCAGCTGTCGCTCCATCGACAAGTTCGCTCAGGGGTGGGGTCCCCTCAC
TTACACTGAGCCTAACAGCTCGGACCAGAGGCCCTACTGCTGGCACTACGCGCCTCG
ACCGTGTGGTATTGTACCCGCGTCTCAGGTGTGCGGTCCAGTGTATTGCTTCACCCCG
AGCCCTGTTGTGGTGGGGACGACCGATCGGTTTGGTGTCCCCACGTATAACTGGGGGG
CGAACGACTCGGATGTGCTGATTCTCAACAACACGCGGNCGCCGCGAGGCAACTGGT
TCGGCTGTACATGGATGAATGGCACTGGGTTCACCAAGACGTGTGGGGGCCCCCCGT
GCAACATCGGGGGGGCCGGCAACAACACCTTGACCTGCCCCACTGACTGTTTTCGGA
AGCACCCCGAGGCCACCTACGCCAGATGCGGTTCTGGGCCCTGGCTGACACCTAGGT
GTATGGTTCATTACCCATATAGGCTCTGGCACTACCCCTGCACTGTCAACTTCACCAT

CTTCAAGGTTAGGATGTACGTGGGGGGCGTGGAGCACAGGTTCGAAGCCGCATGCAA
TTGGACTCGAGGAGAGCGTTGTGACTTGGAGGACAGGGATAGATCAGAGCTTAGCCC
GCTGCTGCTGTCTACAACAGAGTGGCAGATACTGCCCTGTTCCTTCACCACCCTGCCG
GCCCTATCCACCGGCCTGATCCACCTCCATCAGAACATCGTGGACGTGCAATACCTGT
ACGGTGTAGGGTCGGCGGTTGTCTCCCTTGTCATCAAATGGGAGTATGTCCTGTTGCT
CTTCCTTCTCCTGGCAGACGCGCGCATCTGCGCCTGCTTATGGATGATGCTGCTGATA
GCTCAAGCTGAGGCCGCCTTAGAGAACCTGGTGGTCCTCAATGCGGCGGCCGTGGCC
GGGGCGCATGGCACTCTTTCCTTCCTTGTGTTCTTCTGTGCTGCCTGGTACATCAAGGG
CAGGCTGGTCCCTGGTGCGGCATACGCCTTCTATGGCGTGTGGCCGCTGCTCCTGCTT
CTGCTGGCCTTACCACCACGAGCTTATGCCTAGTAA

SEQ ID NO. 24 (HCCI41)
GATCCCACAAGCTGTCGTGGACATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGGG
CCTCGCCTACTATTCCATGGTGGGGAACTGGGCTAAGGTTTTGGTTGTGATGCTACTCT
TTGCCGGCGTCGACGGGCATACCCGCGTGTCAGGAGGGGCAGCAGCCTCCGATACCA
GGGGCCTTGTGTCCCTCTTTAGCCCCGGGTCGGCTCAGAAAATCCAGCTCGTAAACAC
CAACGGCAGTTGGCACATCAACAGGACTGCCCTGAACTGCAACGACTCCCTCCAAAC
AGGGTTCTTTGCCGCACTATTCTACAAACACAAATTCAACTCGTCTGGATGCCCAGAG
CGCTTGGCCAGCTGTCGCTCCATCGACAAGTTCGCTCAGGGGTGGGGTCCCCTCACTT
ACACTGAGCCTAACAGCTCGGACCAGAGGCCCTACTGCTGGCACTACGCGCCTCGAC
CGTGTGGTATTGTACCCGCGTCTCAGGTGTGCGGTCCAGTGTATTGCTTCACCCCGAG
CCCTGTTGTGGTGGGGACGACCGATCGGTTTGGTGTCCCCACGTATAACTGGGGGGCG
AACGACTCGGATGTGCTGATTCTCAACAACACGCGGNCGCCGCGAGGCAACTGGTTC
GGCTGTACATGGATGAATGGCACTGGGTTCACCAAGACGTGTGGGGGCCCCCCGTGC
AACATCGGGGGGGCCGGCAACAACACCTTGACCTGCCCCACTGACTGTTTTCGGAAG
CACCCCGAGGCCACCTACGCCAGATGCGGTTCTGGGCCCTGGCTGACACCTAGGTGT
ATGGTTCATTACCCATATAGGCTCTGGCACTACCCCTGCACTGTCAACTTCACCATCTT
CAAGGTTAGGATGTACGTGGGGGGCGTGGAGCACAGGTTCGAAGCCGCATGCAATTG
GACTCGAGGAGAGCGTTGTGACTTGGAGGACAGGGATAGATCAGAGCTTAGCCCGCT
GCTGCTGTCTACAACAGAGTGGCAGAGTGGCAGAGCTTAATTAATTAG

SEQ ID NO. 25 (HCCI42)
GATCCCACAAGCTGTCGTGGACATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGGG
CCTCGCCTACTATTCCATGGTGGGGAACTGGGCTAAGGTTTTGGTTGTGATGCTACTCT
TTGCCGGCGTCGACGGGCATACCCGCGTGTCAGGAGGGGCAGCAGCCTCCGATACCA
GGGGCCTTGTGTCCCTCTTTAGCCCCGGGTCGGCTCAGAAAATCCAGCTCGTAAACAC
CAACGGCAGTTGGCACATCAACAGGACTGCCCTGAACTGCAACGACTCCCTCCAAAC
AGGGTTCTTTGCCGCACTATTCTACAAACACAAATTCAACTCGTCTGGATGCCCAGAG
CGCTTGGCCAGCTGTCGCTCCATCGACAAGTTCGCTCAGGGGTGGGGTCCCCTCACTT
ACACTGAGCCTAACAGCTCGGACCAGAGGCCCTACTGCTGGCACTACGCGCCTCGAC
CGTGTGGTATTGTACCCGCGTCTCAGGTGTGCGGTCCAGTGTATTGCTTCACCCCGAG
CCCTGTTGTGGTGGGGACGACCGATCGGTTTGGTGTCCCCACGTATAACTGGGGGGCG
AACGACTCGGATGTGCTGATTCTCAACAACACGCGGNCGCCGCGAGGCAACTGGTTC
GGCTGTACATGGATGAATGGCACTGGGTTCACCAAGACGTGTGGGGGCCCCCCGTGC
AACATCGGGGGGGCCGGCAACAACACCTTGACCTGCCCCACTGACTGTTTTCGGAAG
CACCCCGAGGCCACCTACGCCAGATGCGGTTCTGGGCCCTGGCTGACACCTAGGTGT
ATGGTTCATTACCCATATAGGCTCTGGCACTACCCCTGCACTGTCAACTTCACCATCTT
CAAGGTTAGGATGTACGTGGGGGGCGTGGAGCACAGGTTCGAAGCCGCATGCAATTG
GACTCGAGGAGAGCGTTGTGACTTGGAGGACAGGGATAGATCAGAGCTTAGCCCGCT
GCTGCTGTCTACAACAGGTGATCGAGGGCAGACACCATCACCACCATCACTAATAG

SEQ ID NO. 26 (HCCI43)
ATGGTGGGGAACTGGGCTAAGGTTTTGGTTGTGATGCTACTCTTTGCCGGCGTCGACG

GGCATACCCGCGTGTCAGGAGGGGCAGCAGCCTCCGATACCAGGGGCCTTGTGTCCC
TCTTTAGCCCCGGGTCGGCTCAGAAAATCCAGCTCGTAAACACCAACGGCAGTTGGC
ACATCAACAGGACTGCCCTGAACTGCAACGACTCCCTCCAAACAGGGTTCTTTGCCGC
ACTATTCTACAAACACAAATTCAACTCGTCTGGATGCCCAGAGCGCTTGGCCAGCTGT
CGCTCCATCGACAAGTTCGCTCAGGGGTGGGGTCCCCTCACTTACACTGAGCCTAACA
GCTCGGACCAGAGGCCCTACTGCTGGCACTACGCGCCTCGACCGTGTGGTATTGTACC
CGCGTCTCAGGTGTGCGGTCCAGTGTATTGCTTCACCCCGAGCCCTGTTGTGGTGGGG
ACGACCGATCGGTTTGGTGTCCCCACGTATAACTGGGGGGCGAACGACTCGGATGTG
CTGATTCTCAACAACACGCGGNCGCCGCGAGGCAACTGGTTCGGCTGTACATGGATG
AATGGCACTGGGTTCACCAAGACGTGTGGGGGCCCCCCGTGCAACATCGGGGGGGCC
GGCAACAACACCTTGACCTGCCCCACTGACTGTTTTCGGAAGCACCCCGAGGCCACCT
ACGCCAGATGCGGTTCTGGGCCCTGGCTGACACCTAGGTGTATGGTTCATTACCCATA
TAGGCTCTGGCACTACCCCTGCACTGTCAACTTCACCATCTTCAAGGTTAGGATGTAC
GTGGGGGGCGTGGAGCACAGGTTCGAAGCCGCATGCAATTGGACTCGAGGAGAGCGT
TGTGACTTGGAGGACAGGGATAGATCAGAGCTTAGCCCGCTGCTGCTGTCTACAACA
GAGTGGCAGAGCTTAATTAATTAG

SEQ ID NO. 27 (HCCI44)
ATGGTGGGGAACTGGGCTAAGGTTTTGGTTGTGATGCTACTCTTTGCCGGCGTCGACG
GGCATACCCGCGTGTCAGGAGGGGCAGCAGCCTCCGATACCAGGGGCCTTGTGTCCC
TCTTTAGCCCCGGGTCGGCTCAGAAAATCCAGCTCGTAAACACCAACGGCAGTTGGC
ACATCAACAGGACTGCCCTGAACTGCAACGACTCCCTCCAAACAGGGTTCTTTGCCGC
ACTATTCTACAAACACAAATTCAACTCGTCTGGATGCCCAGAGCGCTTGGCCAGCTGT
CGCTCCATCGACAAGTTCGCTCAGGGGTGGGGTCCCCTCACTTACACTGAGCCTAACA
GCTCGGACCAGAGGCCCTACTGCTGGCACTACGCGCCTCGACCGTGTGGTATTGTACC
CGCGTCTCAGGTGTGCGGTCCAGTGTATTGCTTCACCCCGAGCCCTGTTGTGGTGGGG
ACGACCGATCGGTTTGGTGTCCCCACGTATAACTGGGGGGCGAACGACTCGGATGTG
CTGATTCTCAACAACACGCGGNCGCCGCGAGGCAACTGGTTCGGCTGTACATGGATG
AATGGCACTGGGTTCACCAAGACGTGTGGGGGCCCCCCGTGCAACATCGGGGGGGCC
GGCAACAACACCTTGACCTGCCCCACTGACTGTTTTCGGAAGCACCCCGAGGCCACCT
ACGCCAGATGCGGTTCTGGGCCCTGGCTGACACCTAGGTGTATGGTTCATTACCCATA
TAGGCTCTGGCACTACCCCTGCACTGTCAACTTCACCATCTTCAAGGTTAGGATGTAC
GTGGGGGGCGTGGAGCACAGGTTCGAAGCCGCATGCAATTGGACTCGAGGAGAGCGT
TGTGACTTGGAGGACAGGGATAGATCAGAGCTTAGCCCGCTGCTGCTGTCTACAACA
GGTGATCGAGGGCAGACACCATCACCACCATCACTAATAG

SEQ ID NO. 28 (HCCL64)
ATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGGGCCTCGCCTACTATTCCATGGTGG
GGAACTGGGCTAAGGTTTTGGTTGTGATGCTACTCTTTGCCGGCGTCGACGGGCATAC
CCGCGTGTCAGGAGGGGCAGCAGCCTCCGATACCAGGGGCCTTGTGTCCCTCTTTAGC
CCCGGGTCGGCTCAGAAAATCCAGCTCGTAAACACCAACGGCAGTTGGCACATCAAC
AGGACTGCCCTGAACTGCAACGACTCCCTCCAAACAGGGTTCTTTGCCGCACTATTCT
ACAAACACAAATTCAACTCGTCTGGATGCCCAGAGCGCTTGGCCAGCTGTCGCTCCAT
CGACAAGTTCGCTCAGGGGTGGGGTCCCCTCACTTACACTGAGCCTAACAGCTCGGA
CCAGAGGCCCTACTGCTGGCACTACGCGCCTCGACCGTGTGGTATTGTACCCGCGTCT
CAGGTGTGCGGTCCAGTGTATTGCTTCACCCCGAGCCCTGTTGTGGTGGGGACGACCG
ATCGGTTTGGTGTCCCCACGTATAACTGGGGGGCGAACGACTCGGATGTGCTGATTCT
CAACAACACGCGGNCGCCGCGAGGCAACTGGTTCGGCTGTACATGGATGAATGGCAC
TGGGTTCACCAAGACGTGTGGGGGCCCCCCGTGCAACATCGGGGGGGCCGGCAACAA
CACCTTGACCTGCCCCACTGACTGTTTTCGGAAGCACCCCGAGGCCACCTACGCCAGA
TGCGGTTCTGGGCCCTGGCTGACACCTAGGTGTATGGTTCATTACCCATATAGGCTCT
GGCACTACCCCTGCACTGTCAACTTCACCATCTTCAAGGTTAGGATGTACGTGGGGGG
CGTGGAGCACAGGTTCGAAGCCGCATGCAATTGGACTCGAGGAGAGCGTTGTGACTT

GGAGGACAGGGATAGATCAGAGCTTAGCCCGCTGCTGCTGTCTACAACAGAGTGGCA
GATACTGCCCTGTTCCTTCACCACCCTGCCGGCCCTATCCACCGGCCTGATCCACCTC
CATCAGAACATCGTGGACGTGCAATACCTGTACGGTGTAGGGTCGGCGGTTGTCTCCC
TTGTCATCAAATGGGAGTATGTCCTGTTGCTCTTCCTTCTCCTGGCAGACGCGCGCATC
TGCGCCTGCTTATGGATGATGCTGCTGATAGCTCAAGCTGAGGCCGCCTTAGAGAACC
TGGTGGTCCTCAATGCGGCGGCCGTGGCCGGGGCGCATGGCACTCTTTCCTTCCTTGT
GTTCTTCTGTGCTGCCTGGTACATCAAGGGCAGGCTGGTCCCTGGTGCGGCATACGCC
TTCTATGGCGTGTGGCCGCTGCTCCTGCTTCTGCTGGCCTTACCACCACGAGCTTATGC
CTAGTAA

SEQ ID NO. 29 (HCCI65)
AATTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTGGGGTACA
TTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCATGGCGTCC
GGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGCTCTTTCTC
TATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTATGAAGTGC
GCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGCATTGTGTA
TGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAA
CAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGAACGCCAG
CGTCCCCACCACGACAATACGACGCCACGTCGATTTGCTCGTTGGGGCGGCTGCTTTC
TGTTCCGCTATGTACGTGGGGGACCTCTGCGGATCTGTCTTCCTCGTCTCCCAGCTGTT
CACCATCTCGCCTCGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAATCTATCCC
GGCCACATAACGGGTCACCGTATGGCTTGGGATATGATGATGAACTGGTCGCCTACA
ACGGCCCTGGTGGTATCGCAGCTGCTCCGGATCCCACAAGCTGTCGTGGACATGGTGG
CGGGGGCCCATTGGGGAGTCCTGGCGGGCCTCGCCTACTATTCCATGGTGGGGAACT
GGGCTAAGGTTTTGGTTGTGATGCTACTCTTTGCCGGCGTCGACGGGCATACCCGCGT
GTCAGGAGGGGCAGCAGCCTCCGATACCAGGGGCCTTGTGTCCCTCTTTAGCCCCGG
GTCGGCTCAGAAAATCCAGCTCGTAAACACCAACGGCAGTTGGCACATCAACAGGAC
TGCCCTGAACTGCAACGACTCCCTCCAAACAGGGTTCTTTGCCGCACTATTCTACAAA
CACAAATTCAACTCGTCTGGATGCCCAGAGCGCTTGGCCAGCTGTCGCTCCATCGACA
AGTTCGCTCAGGGGTGGGGTCCCCTCACTTACACTGAGCCTAACAGCTCGGACCAGA
GGCCCTACTGCTGGCACTACGCGCCTCGACCGTGTGGTATTGTACCCGCGTCTCAGGT
GTGCGGTCCAGTGTATTGCTTCACCCCGAGCCCTGTTGTGGTGGGGACGACCGATCGG
TTTGGTGTCCCCACGTATAACTGGGGGGCGAACGACTCGGATGTGCTGATTCTCAACA
ACACGCGGNCGCCGCGAGGCAACTGGTTCGGCTGTACATGGATGAATGGCACTGGGT
TCACCAAGACGTGTGGGGGCCCCCCGTGCAACATCGGGGGGGCCGGCAACAACACCT
TGACCTGCCCCACTGACTGTTTTCGGAAGCACCCCGAGGCCACCTACGCCAGATGCG
GTTCTGGGCCCTGGCTGACACCTAGGTGTATGGTTCATTACCCATATAGGCTCTGGCA
CTACCCCTGCACTGTCAACTTCACCATCTTCAAGGTTAGGATGTACGTGGGGGGCGTG
GAGCACAGGTTCGAAGCCGCATGCAATTGGACTCGAGGAGAGCGTTGTGACTTGGAG
GACAGGGATAGATCAGAGCTTAGCCCGCTGCTGCTGTCTACAACAGAGTGGCAGATA
CTGCCCTGTTCCTTCACCACCCTGCCGGCCCTATCCACCGGCCTGATCCACCTCCATC
AGAACATCGTGGACGTGCAATACCTGTACGGTGTAGGGTCGGCGGTTGTCTCCCTTGT
CATCAAATGGGAGTATGTCCTGTTGCTCTTCCTTCTCCTGGCAGACGCGCGCATCTGC
GCCTGCTTATGGATGATGCTGCTGATAGCTCAAGCTGAGGCCGCCTTAGAGAACCTGG
TGGTCCTCAATGCGGCGGCCGTGGCCGGGGCGCATGGCACTCTTTCCTTCCTTGTGTT
CTTCTGTGCTGCCTGGTACATCAAGGGCAGGCTGGTCCCTGGTGCGGCATACGCCTTC
TATGGCGTGTGGCCGCTGCTCCTGCTTCTGCTGGCCTTACCACCACGAGCTTATGCCT
AGTAAGCTT

SEQ ID NO. 30 (HCCI66)
ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCCA
CAGGACGTCAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGC
GCAGGGGCCCCAGGTTGGGTGTGCGCGCGACTAGGAAGACTTCCGAGCGGTCGCAAC

CTCGTGGGAGGCGACAACCTATCCCCAAGGCTCGCCGACCCGAGGGTAGGGCCTGGG
CTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCATGGGGTGGGCAGGAT
GGCTCCTGTCACCCCGCGGCTCTCGGCCTAGTTGGGGCCCTACAGACCCCCGGCGTAG
GTCGCGTAATTTGGGTAAGGTCATCGATACCCTTACATGCGGCTTCGCCGACCTCGTG
GGGTACATTCCGCTCGTCGGCGCCCCCCTAGGGGGCGCTGCCAGGGCCCTGGCGCAT
GGCGTCCGGGTTCTGGAGGACGGCGTGAACTATGCAACAGGGAATTTGCCCGGTTGC
TCTTTCTCTATCTTCCTCTTGGCTTTGCTGTCCTGTCTGACCGTTCCAGCTTCCGCTTAT
GAAGTGCGCAACGTGTCCGGGATGTACCATGTCACGAACGACTGCTCCAACTCAAGC
ATTGTGTATGAGGCAGCGGACATGATCATGCACACCCCCGGGTGCGTGCCCTGCGTTC
GGGAGAACAACTCTTCCCGCTGCTGGGTAGCGCTCACCCCCACGCTCGCAGCTAGGA
ACGCCAGCGTCCCCACCACGACAATACGACGCCACGTCGATTTGCTCGTTGGGGCGG
CTGCTTTCTGTTCCGCTATGTACGTGGGGGACCTCTGCGGATCTGTCTTCCTCGTCTCC
CAGCTGTTCACCATCTCGCCTCGCCGGCATGAGACGGTGCAGGACTGCAATTGCTCAA
TCTATCCCGGCCACATAACGGGTCACCGTATGGCTTGGGATATGATGATGAACTGGTC
GCCTACAACGGCCCTGGTGGTATCGCAGCTGCTCCGGATCCCACAAGCTGTCGTGGAC
ATGGTGGCGGGGGCCCATTGGGGAGTCCTGGCGGGCCTCGCCTACTATTCCATGGTGG
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